DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR ESTIMATION OF METFORMIN AND ALOGLIPTIN IN COMBINED DOSAGE FORM AND FROM PLASMA

A Dissertation submitted to

THE TAMILNADU Dr.M.G.R. MEDICAL UNIVERSITY,

Chennai - 600 032

In partial fulfillment of the requirements for the award of the Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted by

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CERTIFICATE

This is to certify that the dissertation work entitled **"Development and Validation of Analytical Methods for Estimation of Metformin and Alogliptin in Combined Dosage Form and from Plasma"** submitted by **University Reg. No. 261530903** is a bonafide work carried out by the candidate under the guidance of **Dr.S.Malathi, M.Pharm, Ph.D.,** and submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmaceutical Analysis** at the Department of Pharmaceutical Analysis, PSG College of Pharmacy, Coimbatore, during the academic year 2016-2017.

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DECLARATION

I do hereby declare that the dissertation work entitled **"Development and Validation of Analytical Methods for Estimation of Metformin and Alogliptin in Combined Dosage Form and from Plasma"** submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmaceutical Analysis,** was done by me under the guidance of **Dr. S.Malathi, M.Pharm, Ph.D.,** at the Department of Pharmaceutical Analysis, PSG College of Pharmacy, Coimbatore, during the academic year 2016-2017.

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EVALUATION CERTIFICATE

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Examination Center: PSG College of Pharmacy, Coimbatore.

Date:

Internal Examiner **External Examiner**

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Words can never express my sense of gratitude for the unconditional love, support, encouragement and care that my beloved Parents and Sister, continue to shower me with and which has enable me to accomplish and be what I am today.

Above all, I humbly submit our dissertation work, into the hands of Almighty GOD, who is the source of all wisdom and knowledge for the successful completion of our thesis.

I owe more than words can express and for which nothing in this world can ever pay off.

ABBREVIATIONS

RP-HPLC	Reverse phase high performance liquid chromatography
UV	Ultra Violet
HPTLC	High performance thin layer chromatography
nm	Nanometer
Rt	Retention time
R_f	Retention factor
RSD	Relative standard deviation
ICH	International conference harmonization
CV	Correlation coefficient
%	Percentage
mg	milligram
µg	microgram
ng	nanogram
ml	milliliter
v/v	volume by volume
pH	Hydrogen ion concentration
min	minutes
MET	Metformin
ALO	Alogliptin
LC-MS	Liquid chromatography- Mass spectroscopy
LOD	Limit of detection
LOQ	Limit of quantification
PDA	Photo diode array detector

TABLE OF CONTENT

INTRODUCTION

Pharmaceutical Analysis

Pharmaceutical analysis determines the quality of drug products via analytical chemistry. This will introduce areas such as method validation, handling of raw materials and finished products, documentations, inspections that impact the development of pharmaceutical products.

Throughout this, critical cGMPs regulations, FDA guidance and ICH quality guidelines will be discussed specially emphasizing procedures to help individuals to maintain a high level of compliance that rounds the laboratory environment.

Importance of pharmaceutical analysis

- Identify of the drug in formulated product
- Determination of active ingredient or additional impurities
- Stability of the drug
- Rate of drug from its formulation
- Identify and purity of pure drug that meet specification
- Concentration of specified impurities
- Concentration of drug in plasma or biological fluids
- Determine pka values partition coefficients, solubilities and stability of drug under development.

Chemistry

Chemistry is the study of matter including its composition, structure, physical properties and reactivity. There are many approaches to studying chemistry. They were divided into five fields.

They were divided into five fields

1. Organic chemistry
2. Inorganic chemistry
3. Physical chemistry
4. Biochemical chemistry
5. Analytical chemistry

Analytical chemistry

Analytical chemistry is the study of separation, identification and quantification of the chemical components of natural and artificial materials.

Qualitative analysis

Qualitative analysis gives an indication of the identify of the chemical species in the sample.

Quantitative analysis

Quantitative analysis determines the amount of one (or) more of there components. The separation of the component is often performed prior to analysis.

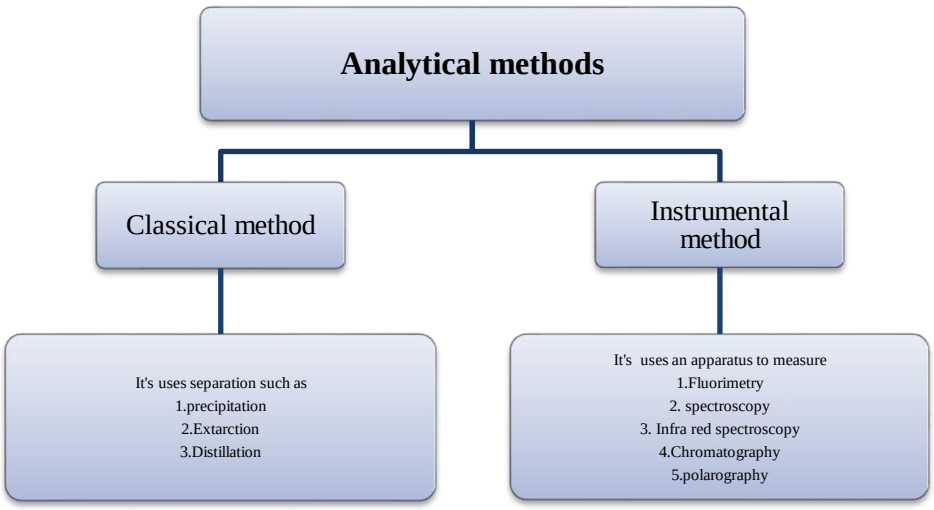
Analytical perspective

Perspective as an analytical approach to solving problems.

- ✓ Identify and define the problem
- ✓ Design the experimental procedure
- ✓ Conduct an experiment and gather data
- ✓ Analyse the experiment data
- ✓ Propose a solution to the problem

Analytical methods

Analytical methods are divided into two types [3]



Instrumental method

It's use an apparatus to measure physical quantities of the analyte such as light absorption, fluorescence and conductivity. Analytical measurements are made using instruments.

Characteristic properties	Instrumental methods
Emission of radiation	Emission spectroscopy, fluorescence, Phosphorescence and luminescence
Absorption of radiation	Spectrometry, Photometry, Nuclear magnetic resonance spectroscopy and Electron spin resonance spectroscopy, infra red spectroscopy
Scattering of radiation	Turbidimetry and Raman spectrometry
Refraction of radiation	Refractometry
Electrical current	Amperometry, polarography
Mass to charge ratio	Mass spectrometry
Electrical potential	Polarography

Application of instrumental methods

1. Bioanalytical:

   Biological molecules and/or biological matrices (e.g. proteins, amino acids, blood, urine)

2. Environmental:

   Pesticides, pollution, air, water, soil.

3. Material science:

   Polymers, Characterisation of new materials.

4. Forensic science:

   Body fluids, DNA, Hair fibres, Elemental analysis, Drugs and Alcohols.

SPECTROSCOPY

Spectroscopy is the branch of science that deals with the interaction between ELECTROMAGNETIC RADIATION and MATTER.

Principles of spectroscopy

The principle is based on the measurement of spectrum of sample containing atoms/molecules. Spectrum is the graph of intensity of absorbed or emitted radiation by sample Vs frequency or wavelength.[3]

Types of Spectroscopy

1. **Absorption spectroscopy**

   An analytical technique which concerns with the measurement of absorption of electromagnetic radiation.

   Eg: UV (185-400nm)/ VISIBLE (400-800nm) spectroscopy

2. **Emission spectroscopy**

   An analytical technique which emission & the amount of dispersion is measured.

   Eg: Flame emission spectroscopy

ULTRAVIOLET AND VISIBLE SPECTROSCOPY

Ultraviolet (UV) spectroscopy is a physical technique of the optical spectroscopy that uses light in the visible, ultraviolet, and near infrared ranges. The Beer-Lambert law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length. Thus, for a fixed path length, UV/VIS spectroscopy can be used to determine the concentration of the absorber in a solution. It is necessary to know how rapidly the absorbance changes with concentration.

Principle of Ultraviolet and Visible Spectroscopy

A molecule or ion will exhibit absorption in the visible or ultraviolet region when radiation causes an electronic transition within its structure. Thus, the absorption of light by a sample in the ultraviolet or visible region is accompanied by a change in the electronic state of the molecules in the sample. The energy supplied by the light will promote electrons from their ground state orbitals to higher energy, excited state orbitals or antibonding orbitals.

Potentially, three types of ground state orbitals may be involved:

1. σ (bonding) molecular

2. π (bonding) molecular orbital

3. n (non-bonding) atomic orbital In addition,

Two types of antibonding orbitals may be involved in the transition:

i) σ* (sigma star) orbital

ii) ii) π* (pi star) orbital

(There is no such thing as an n* antibonding orbital as the n electrons do not form bonds).

Ultraviolet Absorption Spectrophotometry

Spectrophotometry is generally preferred especially by small-scale industries as the cost of the equipment is less and the maintenance problems are minimal. The method of analysis is based on measuring the absorption of a monochromatic light by colourless compounds in the near ultraviolet path of spectrum (200-380nm). The photometric methods of analysis are based on the Bouger-Lambert-Beer's law, which establishes that the absorbance of a solution is directly proportional to the concentration of the analyte.

The fundamental principle of operation of spectrophotometer covering UV region consists in that light of definite interval of wavelength passes through a cell with solvent and falls on to the photoelectric cell that transforms the radiant energy into electrical energy measured by a galvanometer. Ultraviolet visible spectroscopy is used to obtain the absorbance spectra of a compound in solution or as a solid. What is actually being observed spectroscopically is the absorbance of light energy or electromagnetic radiation, which excites electrons from the ground state to the first singlet excited state of the compound or material. The UV-visible region of energy for the electromagnetic spectrum covers 1.5 - 6.2 eV which relates to a wavelength range of 800 - 200 nm.

The Beer-Lambert Law (Equation1) is the principle behind absorbance spectroscopy. For a single wavelength, A is absorbance (unit less, usually seen as arb. units or arbitrary units), is the molar absorptivity of the compound or molecule in solution (M-1cm-1), b is the path length of the cuvette or sample holder (usually 1 cm), and c is the concentration of the solution (M).

$$A = a\ b\ c$$

Where,

A = Absorbance,

a = absorptivity,

b = path length,

c = concentration.

$$C = A / a\ b$$

There are three types of absorbance instruments used to collect UV-Visible spectra:

1. Single beam spectrometer.
2. Double beam spectrometer.

All of these instruments have a light source (usually a deuterium or tungsten lamp), a sample holder and a detector, but some have a filter for selecting one wavelength at a time. The single beam instrument has a filter or a monochromator between the source and the sample to analyze one wavelength at a time. The double beam instrument has a single source and a monochromator and then there is a splitter and a series of mirrors to get the beam to a reference sample and the sample to be analyzed, this allows for more accurate monochromator between the sample and the source. Instead, it has a diode array detector that allows the instrument to simultaneously detect the absorbance at all wavelengths. The simultaneous instrument is usually much faster and more efficient, but all of these types of spectrometers work well.

Multicomponent analysis is pharmaceutical dosage forms by UV spectrophotometry

The spectroscopic assay of drug rarely involves the measurement of absorbance of samples containing only one absorbing component. The pharmaceutical analyst frequently encounters the situation where the concentration of the or more substances is required in samples known to contain other absorbing substances which potentially interfere in the assay it the formula of the samples is known of the samples is known the identify and concentration of the interfere are known and the extent of interference in assay may be determined.

Some of the commonly used spectrophotometric methods are as follows

1. Simultaneous equation method (vierdott's method)
2. Derivative spectrometric method
3. Absorbance ratio method(Q-Absorbance method)
4. Solvent extraction method
5. Dual wavelength method
6. Geometric correction method
7. H.point standard addition method
8. Least square approximation method

SIMULTANEOUS EQUATION METHOD

If a sample contain two absorbing drugs (X &Y) each of this absorbs at the λ_{max} of each other, it may be possible to determine both the drugs by the technique of simultaneous equations (vierodt's method) provided that certain criteria apply.

The information required is:

(a) the absorptivities of X at λ_1 and λ_2, ax_1 and ax_2 respectively

(b) the absorptivities of Y at λ_1 and λ_2, ay_1 and ay_2 respectively

(c) the absorbance of the diluted sample at λ_1 and λ_2, A_1 and A_2 respectively.

Let c_x & c_y be the concentrations of X & Y respectively in the diluted sample.

Two equations are constructed based upon the fact that at λ_1 and λ_2 the absorbance of the mixture is the sum of the individual absorbances of X&Y.

At λ_1 $A_1 = ax_1bc_x + ay_1bc_y$ ----------1

At λ_2 $A_2 = ax_2bc_x + ay_2bc_y$----------2

For measurements in 1cm cells, b=1.

Rearrange the eq. (2).

$$c_y = \frac{A_2 - ax_2c_x}{ay_2}$$

Substituting for C_y in eq. (1) and rearranging gives

$$c_x = \frac{A_2\, ay_1 - A_1\, ay_2}{ax_2\, ay_1 - ax_1\, ay_2}$$

and

$$c_y = \frac{A_1\, ax_2 - A_2\, ax_1}{ax_2\, ay_1 - ax_1\, ay_2}$$

Criteria for obtaining maximum precision based upon the absorbance ratio have been suggested that place limit on the relative concentration of the components of the mixture. The criteria are that of the ratio

$$\frac{A_2/A_1}{ax_2/ax_1} \quad \text{and} \quad \frac{ay_2/ay_1}{A_2/A_1}$$

Where,

c_x and c_y are the concentrations of X and Y.

ax_1 and ax_2 are absorptivities of X.

ay_1 and ay_2 are absorptivities of Y.

A_1 and A_2 absorbances of mixture.

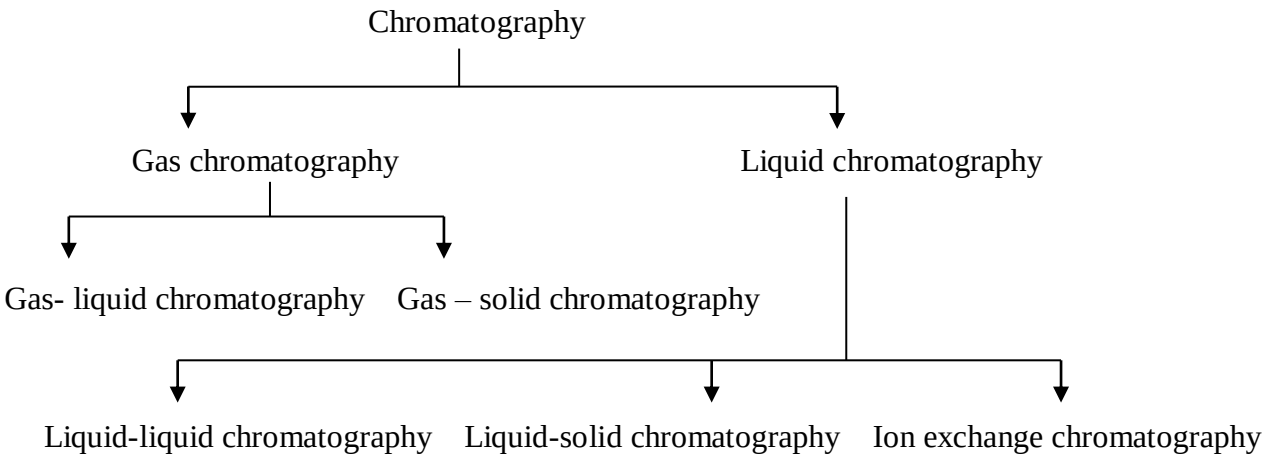
Chromatography

Chromatography is the powerful techniques in which differential migration of components takes place between two phases, one is stable which is known as stationary phase and another is movable which is known as mobile phase. Species in the sample undergo repeated interaction (partition) between the mobile phase and stationary phase.

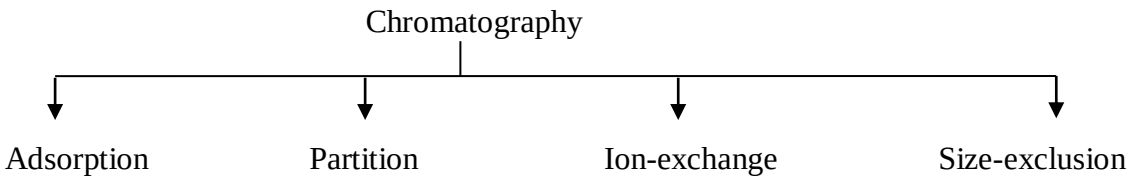
Chromatographic method must having essentially,

- Stationary phase
- Mobile phase
- Sample injection system
- Solvent delivery system
- Column (support for stationary phase)
- Detection by detecting agent

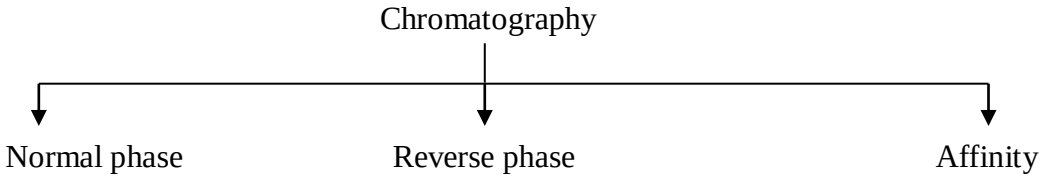
Classification of chromatographic methods



Types of chromatography (Based on nature)



Chromatography (Based on separation)



HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

High performance thin layer chromatography is the most powerful advanced form of Thin Layer Chromatography (TLC) and consists of chromatographic layers of utmost separation efficiency and the application of sophisticated instrumentation for all steps in the procedure include accurate sample application, standardized reproducible chromatogram development and software controlled evaluation.

Advantages of HPTLC

- ✓ The separation process is easy to follow especially with colored compounds.
- ✓ Choice of solvents for HPTLC method development is wide as the mobile phases are fully evaporated before the detection step.
- ✓ Two- dimensional separations are easy to perform.
- ✓ HPTLC can combine and consequently be used for different modes of evaluation, allowing identification of compounds having different light absorption characteristics or different colours.
- ✓ HPTLC method may help to minimizes exposure risk of toxic organic effluents and significantly reduces its disposal problems, consequently reduces environmental pollution.

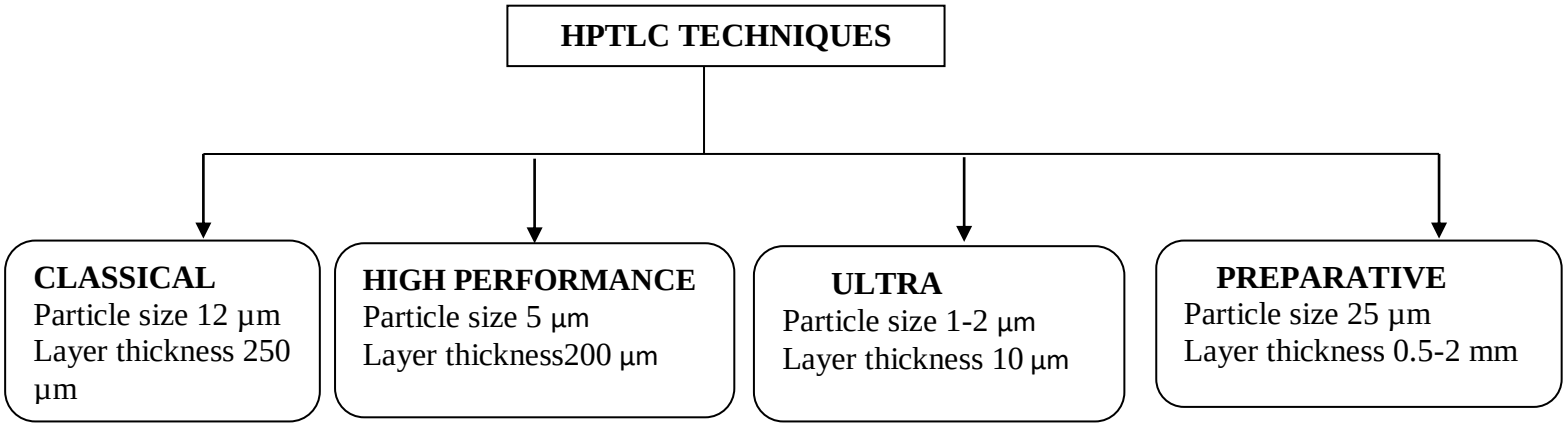
Principle of HPTLC

HPTLC have similar approach and employ the same physical principles of TLC (adsorption chromatography) i.e. the principle of separation is **adsorption.**

The mobile phase solvent flows through because of capillary action. The components move according to their affinities towards the adsorbent. The component with more affinity towards the stationary phase travels slower. The component with lesser affinity towards the stationary phase travels faster. Thus the components are separated on a chromatographic plate.

Classification of HPTLC

HPTLC techniques may be classified into four classes. They differ with classical TLC in the particle size, distribution and thickness of sorbent layers.



Methodology for HPTLC Analysis

Method development in thin- layer (planar) chromatography is one of the most significant steps for a qualitative and quantitative analysis. During establishing a new analytical procedure, always starts with wide literature survey i.e. primary information about the physicochemical properties of the sample and nature sample (structure, polarity, volatility, stability and solubility). It involves considerable trial and error procedures. General steps involved in HPTLC method development are as follows;

Basic steps:

- Selection of the stationary phase
- Mobile phase selection and optimization
- Sample preparation and Application
- Chromatogram development (separation)
- Detection

Selection of stationary phase

During method development, stationary phase selection should be based on the type of compounds to be separated. HPTLC uses smaller plates (10*10 or 10*20 cm) with significantly decreased development distance (typically 6 cm) and analysis time (7-20 mins). HPTLC plates provide improved resolution, higher detection sensitivity and improved in situ quantification and are used for industrial pharmaceutical densitometric quantitative analysis.

Mobile phase selection and optimization

The selection of mobile phase is based on adsorbent material used as stationary phase and physical and chemical properties of analyte. Mobile phase for HPTLC method is selected and optimized on the basis of analyst's own experience, literature report of similar studies and traditional trial and error method. CAMAG laboratory suggested four levels of techniques for mobile phase selection and optimization.

First level involves the use of neat solvents (seven to twelve) and then by finding such solvents which can have average separation power of the desired drugs (optimization stops here if the analytical goal is achieved). Further, at this level the solvents are categorised on the basis of R_f value they produce:

Group A – solvents produce suitable R_f value ($0.2< R_f<0.8$),

Group B – solvent produces R_f value higher than 0.8

Group C – solvents produces R_f value less than 0.2

Second level involves decreasing or increasing solvent strength using hexane or water for respective purposes.

Third level involves trying of mixtures instead of neat solvents from the selected solvents of first and/or second level which can further be optimized by the use of modifier, such as acids or bases.

If the R_f values are acceptable, go for level four, which includes, minor adjustment to the solvent strength, use of modifiers to improve the shape of the separated zones and alteration of chamber saturation time. If the separated zones show tailing, try adding 0.5 to 1.0% water. Try exchanging acid or base for a weaker/stronger one.

Sample Preparation and Application

A good solvent system is one of that moves all components of the mixture off baseline, but does not put anything on the solvent front. The peaks of interest should be resolved between R_f 0.15 and 0.85. The elution power of the mobile phase depends on a property called eluent strength which is related to the polarity of the mobile phase components. The more nonpolar compounds, the faster it will elute (or the less time it will remain on the stationary phase) and the more polar compound slower it will elute (or more time on the stationary phase). Pharmaceutical preparation with sufficiently high concentration of analyte is simply dissolved in a suitable solvent that will completely solubilise the analyte and leave excipients undissolved to yield a test solution that can be directly applied on HPTLC plate. It is a fact that application of the sample is the most critical step to obtain good

resolution for quantification in HPTLC. Sample application technique depends on factors such as the type of sample matrix, workload and time constraints.

Chromatogram development

Although chromatogram development is the most crucial step in the HPTLC procedure, important parameters are generally overlooked. HPTLC plates are developed in twin-trough chambers, or horizontal- development chambers. In general, saturated twin trough chambers fitted with filter paper offer the best reproducibility. Twin trough chamber avoids solvent vapour preloading and humidity.

Detection

Detection of separated compounds on the sorbent layers is enhanced by quenching of fluorescence due to UV light (ranged normally at 200-400 nm). This process is commonly called fluorescence quenching.

Visualization at UV 254 nm

F254 should be described as phosphorescence quenching. In this instance the fluorescence remains for a short period after the source of excitation is removed. It is very short lived, but longer than 10 seconds. F254 fluorescent indicator is excited with UV wavelength at 254 nm and emits green fluorescence. Compounds that absorb radiation at 254 nm reduce the emission on the layer and a dark violet spot on a green background is observed where the compound where the components zones are located.

Visualization at UV 366 nm

F366 should be described as fluorescence quenching. In this instance the fluorescence dose not remains after the source of excitation is removed.

Visualization at white light

Zone containing separated compounds can be detected by viewing their natural colour in daylight (white light).

BIOANALYSIS

Bioanalysis is the method used to determine the concentration of drugs, their metabolites and/or endogenous substance in the biological matrices such as blood, plasma, cerebrospinal fluid, urine and saliva.

Reasons for developing new method of Bioanalysis

- There may not be a suitable method for a particular analyte in the specific sample matrix.
- Existing method may be too error (or) contamination prone (or) they may be unreliable.
- Existing method may be too expensive, time consuming (or) energy intensive (or) they may not be easily automated.
- Existing method may not provide adequate sensitivity (or) analyte selectivity in sample of interest.
- Newer instrumentation and techniques may have evolved that provide opportunities for improved methods including improved analyte identification (or) detection limits, greater accuracy (or) precision or better return on investment.
- There may be need for an alternative method to confirm, for legal (or) scientific reasons, analytical data originally obtained by existing method.

Bioanalytical method

Bioanalytical method is a set of procedures involved in the collection, processing, storage and analysis of a biological matrix for a chemical compound.

Separation of analyte

Extraction procedures for drugs and metabolites from biological samples:

Extraction of drug from biological matrix is traditionally carried out by

a) Liquid-Liquid Extraction(LLE)
b) Solid Phase Extraction (SPE)
c) Precipitation of plasma Proteins (pp)

(a) Liquid-Liquid Extraction

LLE is a method used for the separation of compounds in a mixture using water and an immiscible organic solvent. Separation of analyte occurs based on its partition co-efficient between two immiscible liquids and extraction can be done by using suitable solvent. LLE method is simple, rapid and relatively cost effective compare to other techniques. Most of the drug can be recovered to the extent of 90% by multiple continuous extraction technique.

First dissolve the components mixture in a suitable solvent and add immiscible solvent. Thoroughly mix and set aside. Separation of immiscible solvent into layers. The components of mixture distributed among the solvents based on their partition co-efficient. Separate the two layers transfer and isolate. The non-polar analyte extracted into organic phase and it's easily recovered by evaporation. Then the residue reconstitute with a small volume of mobile phase.

(b) Solid Phase Extraction

SPE is a common and effective technique for isolation and concentration of analyte in trace amounts in a variety of sample matrices. With SPE the level of interferences can be reduced and final sample volume is minimized to maximize analyte sensitivity. Higher recovery of analyte can be obtained by using a small plastic disposable column or cartridge packed with 0.1 to 0.5 g of sorbent which is commonly RP material (C18 or C8). The components of interest may either preferentially adsorbed to the solid or they may remain in the liquid phase. If the desired analyte is adsorbed on the solid phase, it can be selectively desorbed by washing with an appropriate solvent. If the component of interest remains in a liquid phase, it can be recovered through concentration, evaporation and or crystallization.

(c) **Protein Precipitation**

Protein precipitation is a very simple technique for extraction of the analyte from the blood (or) plasma. The main requirement for this technique is that the analyte should be freely soluble into reconstituting solvent. In this technique, the sample is prepared by protein precipitation. Sample is treated with the acids (trichloroacetic acid, perchloric acid)/ Organic solvents (Methanol, Ethanol and acetone)/ Salts (ammonium sulphate). After precipitation the sample is centrifuged, analyte gets into supernatant. The supernatant liquid is injected into the HPLC.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC method development and validation plays an important role in the discovery, manufacturing, development of pharmaceutical products. HPLC methods are able to separate, detect and quantify various drugs their related substances, degraded products and impurities that may be introduced during the synthesis of drug substances.

Biological matrices contain high concentrations of various endogenous substances and drug concentrations are often low. Sometimes the endogenous compounds are structurally similar to the drug to be estimated. For analysis, the drug must be isolated in pure state using a suitable extraction technique. Invention of modern analytical instruments and extraction techniques resulted in advanced bio analytical method development and validation.

High performance liquid chromatography (HPLC) is useful in identification and quantitative determination of drugs and metabolites in biological fluids, particularly plasma, serum or urine. HPLC require good selection of detectors, good stationary phase, Eluents and adequate program during separation. UV/VIS, PDA detectors are the most versatile detector used in HPLC. The main advantages of these chromatographic principles include low detection limits, the ability to generate structural information the requirement of minimal sample treatment and the possibility to cover a wide range of analytes differing in their polarities.

Advantages of HPLC

- Speed (analysis can be accomplished in 20 minutes or less)
- Greater sensitivity (various detectors can be employed)
- Improved resolution (wide variety of stationary phases)
- Reusable columns (expensive columns but can be used for many analysis)
- Ideal for the substances of low volatility
- Easy sample recovery, handling and maintenance
- Instrumentation tends itself to automation and quantitation (less time and less labour)
- Precise and reproducible,
- Calculations are done by integrator itself
- Suitable for preparative liquid chromatography on a much larger scale.

Normal phase mode

The stationary phase is polar and the mobile phase is nonpolar in nature. In this technique, nonpolar compounds travel faster and are eluted first. This is because of the lower affinity between the nonpolar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

Reversed phase mode

It's the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is nonpolar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compound gets eluted first in this mode and nonpolar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are octa decyl silane (ODS) or C18, C8, C4, etc., (in the order of increasing polarity of the stationary phase).

Principle

Principle is the solution of sample is injected into a column of porous material (stationary phase) and liquid phase (mobile phase) is pumped at higher pressure through the column. The principle of separation followed is the adsorption of solute on stationary phase based on its affinity towards stationary phase.

Method development on HPLC

Steps involved in the HPLC method development is as follows

- ✓ Understanding the physicochemical properties of drug molecule
- ✓ Selection of chromatographic conditions
- ✓ Developing the approach of analysis
- ✓ Sample preparation
- ✓ Method optimization
- ✓ Method validation

Instrumentation

Components of HPLC are

(a) Solvent delivery system including pump

(b) Sample injection system

(c) Chromatographic column

(d) Detector

(e) Chart recorder

(f) Data handling device and microprocessor control

(a) **Solvent delivery system**

A mobile phase is pumped under pressure from one or several reservoir and flows through the column at a constant rate. For normal phase separation-eluting power increases with increasing polarity of the solvent but for reverse phase separation, eluting power decreases with increasing polarity. A degasser is needed to remove dissolved air and other gases from the solvent. Special grades of solvents are available for HPLC and these have been purified carefully in order to remove absorbing impurities and particulate matter to prevent these particles from damaging the pumping or injection system or clogging the column.

Pumps

The pump is one of the most important components of HPLC. Since its performance directly affects retention time, reproducibility and detector sensitivity. Three main types of pumps are used in HPLC to propel the liquid mobile phase through the system. These are;

1. **Displacement pump**

It produces a flow that tends to independent of viscosity and backpressure and also output is pulse free. But its possesses limited capacity (250ml).

2. **Reciprocating pump**

It has small internal volume (35 to 400 µl), their high output pressure (up to 10,000 psi) and their constant flow rates, but it produces a pulsed flow.

3. **Pneumatic or constant pressure pump:**
   They are pulse free, suffer from limited capacity as well as dependence of flow rate on solvent viscosity and column backpressure. They are limited to pressure less than 2000 psi.

(b). **Sample injection system**

Insertion of the sample onto the pressurised column must be as a narrow plug so that the peak broadening attributable to this step is negligible. The injection system itself should have no dead (void) volume.

These are three important ways of introducing the sample into injection port:

- Loop injection: In which, a fixed amount of volume is introduced by making use of fixed volume loop injector.
- Valve injection: In which, a variable volume is introduced by making use of an injection valve.
- On column injection: In which, a variable volume is introduced by means of a syringe through a septum.

(c). Chromatographic column:

The column is usually made up of heavy glass or stainless steel tubing to withstand high pressure. The columns are usually 10-30 cm long and 4-10 mm inside diameter containing stationary phase at particle diameter of 25 µm or less. Columns with an internal diameter of 5 mm give good results because of compromise between efficiency, sample capacity and the amount of packing and solvent required.

Column packing

The packing used in modern HPLC consists of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC.

1. **Porous, polymeric beds**

   Porous, polymeric beds based on styrene divinyl benzene co polymers used in ion exchange and size exclusion chromatography. For analytical purpose these have now been replaced by silica based, packing which are more efficient and more stable.

2. **Porous layer beds**

   Consisting of a thin shell (1-3 µm) of silica on an spherical inert core (e.g. glass). After the development of totally porous micro particulate packing, these have not been use in HPLC.

3. **Totally porous silica particles (dia.<10 µm)**

These packing have widely been used for analytical HPLC in recent years. Particles of diameter >20 µm are usually dry packed, while particles of diameter <20 µm are slurry packed in which particles are suspended on a suitable solvent and the slurry so obtained is driven into the column under pressure.

(d) Detectors

A chromatography detector is a device used in high performance liquid chromatography (HPLC) to detect components of the mixture being eluted off the chromatographic column. The detector senses the presence of the individual components as they leave (elute) the column. The detector converts a change into an electric signal that is recorded by data system.

CLASSIFICATION OF HPLC DETECTORS

- UV/VIS
  - Fixed wavelength
  - Variable wavelength
  - Diode array (PDA)
- Refractive index
  - Deflection detector
  - Refractive detector
- Fluorescence detector
- Electrochemical detector
- Conductivity detector
- Evaporative light scattering detector
- Mass detector

ULTAVIOLET/VISIBLE SPECTROSCOPIC DETECTORS

Measures the ability of solute to absorb light at particular wavelength(s) in the ultraviolet (uv) (or) visible (vis) wavelength range. When a light of a certain wavelength is directed at a flow cell, the substance inside the flow cell absorbs the light. As a result, the intensity of the light that leaves the flow cell is less than that of the light that enters it. An absorbance detector measures the extent to which the light intensity decreases (i.e. absorbance).

Fixed wavelength detector

Its absorbance of only one given wavelength is monitored by the system at all times (usually 254 nm)

- Simplest and cheapest of the UV/VIS detector
- Limited flexibility
- Limited in types of compounds that can be monitored

Variable wavelength detector

It a single wavelength detector is monitored at any given time, but any wavelength ina wide spectral range can be selected

- Wavelength vary from 190-900 nm
- More expensive, requires more advanced optics
- More versatile, used for a wider range of compounds
- More sensitive due to photomultiplier tube or amplification circuitry

Photo Diode Array Detector

Operates by simultaneously monitoring absorbance of solutes at several different wavelengths. Light from the broad emission source such as a deuterium lamp is collimated by an achromatic lens system so that the total light passes through detector cell onto a holographic grating. In this way, the sample is subjected to light of all wavelengths generated by lamp. The dispersed light from the grating is allowed to fall on to a diode array. The array may contain many hundreds of diodes and the output from each diode is regularly sampled by a computer and stored on hard disc.

VALIDATION

According to ICH method validation is defined by establishing documented evidence which proves a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specification and quality characteristics.

Validation parameters

- Recovery
- Response function
- Sensitivity
- Precision
- Accuracy
- Limit of detection
- Limit of quantification
- Robustness
- Stability
- System suitability

(a) Recovery

The absolute recovery of analytical method is measured as the response of a processed standard expressed as a percentage of response of pure standard which has not been subjected to sample pre-treatment and indicates whether the method provides a response for the entire amount of analyte that is present in the sample. It is performed by comparing the responses of extracted sample at low, medium and high concentration in replicates of at least 6 with those non-extracted standards which represent 100% recovery.

$$\textbf{\textit{Absolute recovery}} = \frac{\textbf{\textit{Response of analyte}}}{\textbf{\textit{Response of analyte of pure standard}}}$$

(b) Response function

Peak area and peak height may be used as response function to define the linear relationship with concentration known as the calibration model.

(c) Sensitivity

If small changes in the concentration method is said to be cause large changes in response function. The sensitivity of an analytical method is determined from the slope of the calibration line. The limit of quantification or working dynamic range of bio-analytical method is defined as the highest and lowest concentrations, which can be determined with acceptable accuracy. According to this it will be set as ±15 for the upper and lower limit of quantification respectively. Any sample concentration that falls outside the calibration range cannot be interpreted from the calibration line and extrapolation of calibration curve is discouraged. If the concentration is over range, the sample should be diluted in drug-free matrix and re-assayed.

(d) Precision

Precision is done to determine the valid estimation of a true value. It determines the variation in the result when the analytical process is repeated. Precision includes repeatability, intermediate precision and reproducibility. Precision and accuracy together determines the error of an individual determination. (Willard *et.al* 2015)

Precision refers to the reproducibility of measurement with in a set that is the scatter of dispersion of a set about its central value. 'Set' means the number (n) of independent replication measurements of some property. Standard deviation is one of the most popular terms employed.

(e) Accuracy

Accuracy refers to closeness of agreement with the true result. There are two methods for determining accuracy they are absolute method and comparative method. Accuracy is best reported as percentage bias. Accuracy is determined by replicate analysis of samples containing known amounts of analyte. The deviation of the mean from the true value serves as the measure of accuracy.

1. Calibration

When good calibration method is adopted then a good precision and accuracy can be obtained. The most convenient calibration function is linear, goes through the origin and is applicable over a wide dynamic range. Linearity identifies the range of concentrations over which the method response is proportional to the amount of analyte. In practise deviations may ocur from the ideal calibration curve. The major calibration equation used for analytical technique is

$$y = mx + C$$

m = Slope

C = Intercept

In calibration, univariate regression is applied, which means that all observations are dependent upon a single variable x.

2. Standard deviation of slope

The standard deviation of slope is proportional to standard error of estimate and inversely proportional to the range and square root of the number of data points.

3. Correlation coefficient

The correlation coefficient 'r' is more usefull to express the relationship of chosen scales. To obtain the correlation coefficient the co-variance is divided by the product of standard deviation of x and y.

$$r = \frac{1}{n-1} \cdot \sum_{i=1}^{n} \frac{x_i - \bar{x}}{s_x} \cdot \frac{y_i - \bar{y}}{s_y}$$

(f) Limit of detection (LOD)

Limit of detection of an analytical method may be defined as the concentration which gives rise to an instrumental signal that is significantly different from the blank. They mainly depend upon the calibration curve for quantitative measurements, the IUPAC approach employs standard deviation of the intercept (Sa), which may be related to LOD and the slope of the calibration curve 'b' by

$$LOD = 3Sa/b$$

(g) Limit of Quantification (LOQ

Limit of quantification can be quantitative reliably with a specified level of accuracy and precision. LOQ represents the concentration of analyte that would yield a signal to noise ratio of 10.

$$LOQ = 10Sa/b$$

Sa = the estimate is the standard deviation of the peak area ratio of analyte to IS (5 injection) of the drugs.

b = slope of the corresponding calibration curve.

(h) Robustness

According to ICH robustness is the" measure of its capacity to remain unaffected by small but deliberate variations in method parameters". It means the ability of the method to withstand the changes in method, reagents etc... The main parameters are pH of the mobile

phase, temperature, percentage of the organic solvent strength and buffer concentrations etc.to determine the robustness of the mehod experimental conditions were purposely altered and chromatographic characters were evaluated.

(i) Stability

The samples, standards and the reagents used for the HPLC method must be stable for a reasonable time (e.g. one day ,one week, one month depending upon the need) for reproducible and reliable results. The analysis of even a single sample may require ten or more chromatographic runs to determine the system suitability, including standard concentrations to create a working analytical curve and duplicate or triplicate injections of the sample to be assayed.

(j) System suitability

All quality systems which are appropriate to analytical chemistry require that the equipment used is 'fit for purpose'. It requires the performance characteristics of the measurement system meet the requirements of the analytical method.

The system suitability tests includes

I. *Capacity factor(k)*

It gives the fractional delay introduced by the separation process. This is equal to the ratio of the time spent by the analyte in the stationary and mobile phases. The capacity factor will be strongly affected by the solvent composition in HPLC.(Graham Currel)

II. *Asymmetry*

The actual shape for a symmetrical peak can be represented by a Gaussian curve. The asymmetry of the peak can be defined by using the ratio between the forward and tailing half-widths of the peak.

$$A\ 10\% = Y/X$$

III. *Separation factor*

Separation factor is defined as the ratio between the capacity factors for the two components. The separation factor is a function of choice of stationary and mobile phase. It is denoted by α

$$\alpha = k_a/k_b$$

IV. *Column efficiency*

The efficiency of the column is the measure of its ability to produce a peak that is narrow in relation to its retention time. This is measured historically by the plate no (N).[ICH Q_2B ,Q_3B] .[1-10]

LITERATURE REVIEW

1. Kaushelendra mishra *et a*l., (2011) developed and validated metformin hydrochloride in tablet dosage form. The analysis complied with beers law in the concentration range of 8-13 µg/ml at 233 nm for metformin. The validation of analytical method determination of metformin by UV in tablet formulation was performed in accordance the parameter including specificity, LOD, LOQ, Linearity and robustness. The linearity range between 8-13 µg/ml and correlation coefficient value was found to be 0.9991.[11]
2. A Madhukar *et al.,* (2011) developed simple and sensitive method for metformin in RP-HPLC. Separation was achieved an inertsil - extend – C_{18} HPLC column (250 mm x 4.6 mm). 10 mM 1-octane sulfonic acid: Acetonitrile (80:20 v/v) as a mobile phase. The detection was carried out using PDA detector at 232 nm. The linear concentration range was 1-250 µg/ml and correlation co-efficient value was found to be 0.9995.[12]
3. Dhaneswar *et al.,* (2010) developed a method for metformin in bulk drug and formulation by HPTLC. Metformin was chromatographed on silica gel 60 F_{254} TLC plate using ammonium sulphate (0.5 %): 2-propanol: methanol (8: 1.6: 1.6 v/v/v) as mobile phase. R_f value was found to be 0.50 ± 0.03 it's was scanned at 238 nm. The linear regression showed a good relationship with correlation co- efficient 0.999.[13]
4. K Heinig *et al.,* (2004) developed fast liquid chromatographic – tandem mass spectrometric (LC-MS/MS) method for determination of metformin in plasma sample. This method employed a YMC cyano (2 mm x 50 mm , 3 µm) analytical column. For minimum sample preparation direct injection of samples after protein precipitation was performed. The polar column used with high organic mobile phase. A step gradient from 100 % ACN to ACN: Water (80:20 v/v) containing a 10 mM Ammonium acetate and 1% acetic acid. Second method a column switching LC-MS/MS assay for online trapping was developed. Column used as YMC cyano column (250 mm x 10 mm, 5 µm) the mobile phase used as ACN: Methanol (95 :5 v/v). elution was performed isocrtically using 10mM ammonium acetate in ACN: Water (80:20 v/v) 1% formic acid. The linear range 10 – 10,000 ng/ml.[14]

5. H Amini *et al.*, (2005) developed for the determination of antihyperglycemic agent metformin in human plasma using a novel sample extraction procedure. Liquid-liquid extraction of metformin and ranitidine (as internal standard) from plasma samples was performed with 1-butanol/n-hexane (50:50, v/v) in alkaline condition followed by back-extraction into diluted acetic acid. Chromatography was carried out using a silica column (250 mmx4.6 mm, 5 micron) under isocratic elution with acetonitrile-40 mM aqueous sodium dihydrogen phosphate (25:75, v/v), pH 6. The limit of quantification (LOQ) was 15.6 ng/ml and the calibration curves were linear up to 2000 ng/ml.[15]
6. D R Brocks *et al.*, (2010) developed a selective sensitive HPLC method for determination of metformin in human plasma and urine using conventional RP column and low specimen volume. Ranitidine is used as an internal standard and extraction was done form plasma and urine sample was performed with 1- butanol: hexane (50:50 v/v) mixture under alkaline conditions followed by back extraction into dil. acetic acid. Chromatography was carried out using C_{18} column (250 mm x 4.6 mm, 5 µm). ACN: potassium dihydrogen orthophosphate (34:66 v/v) as mobile phase. The flow rate maintained at 0.7 ml/min. the linear regression range found to be 2-2000 µg/ml. The mean absolute recoveries for 100 and 1000 ng /ml metformin hydrochloride in plasma using the present extraction procedure were 93.7 % and 88.5 %, respectively.[16]
7. Chandrabatla varaprasad *et al.*, (2015) developed RP-HPLC method for the metformin and linagliptin using PDA detector at 225 nm. The linear range was 250 – 2500 µg/ml and 1.25 – 12.5 µg/ml for metformin and linagliptin, respectively. Analytical column waters x-bridge C_{18} (500 mm x4.6 mm, 5 µ) was used at temperature of 30ºC ± 0.5ºC. CAN : 0.02 M phosphate buffer (PH-5) in the ratio of 35 :65 v/v were used as mobile phase at a flow rate 1 ml/min. Retention time was 1.64 and 4.6 min for metformin and linagliptin respectively.[17]
8. K Neelema *et al.*, (2014) developed a gradient RP-HPLC method for metformin, voglibose and glimepiride in bulk and combined tablet dosage form. The separation carried out for three drug was based on the use of inertsil ODS 3V (150 mm x 4.6 mm, 5 µm) column in a gradient mode. The mobile phase was consisting of 0.02 m phosphate buffer (PH- 2.5) adjusting with Orthophosphoric acid (solution A) and acetonitrile (solution B). The flow rate was 1ml/min and detection at 230 nm. The retention time 2.423, 8.19 and 11.708 of metformin, voglibose and glimepride,

respectively. The linear for metformin, voglibose and glimepride at concentration range of 200-600 µg/ml, 0.08-0.24 µg/ml and 0.8-2.4 µg/ml.[18]

9. A R Shirode *et al.,* (2014) developed RP-HPLC and HPTLC methods for simultaneous estimation of metformin hydrochloride and vidagliptin from bulk and marketed formulation. The separation was carried out by HiQsil C_{18} HS (250 mm x4.6 mm) column and detection was carried out by using variable wavelength detector. Phosphate buffer: methanol: Acetonitrile (50:30:20 v/v/v) used as mobile phase and flow rate was 0.8 ml/min. metformin and vidagliptin were retention time 3.7 min and 4.8 min, respectively. Linear range was 10-60 µg/ml for metformin and vidagliptin, respectively.[19]
10. G Saravanakumar *et al.,* (2014) developed and validated consistent UPLC method for metformin, pioglitazone and glimepride in tablet dosage form. UPLC separation was achieved on symmetry C_{18} (2.1mm x 100 mm, 1.7 µm) as isocratic mode. Phosphate buffer: methanol (UPLC grade) was used as mobile phase. The flow rate was 0.25 ml/min. The retention time was 0.002 min, 1.77 min and 2.409 min for metformin, pioglitazone and glimepride, respectively. Linearity range was 400-600 ppm for metformin, 12-18 ppm for pioglitazone and 0.8-1.2 ppm for glimepride. The % recovery was found to be 99.7%-100.9%, 98.4%-100.9% and 99.9%-101.2% for metformin, pioglitazone and glimepiride, respectively.[20]
11. F AI- Rimawi (2009), developed and validated an analytical method for metformin and its related compound (1-cyanoguanidine) in tablet formulation by HPLC-UV. Liquid chromatography with UV- detector at a wavelength 232 nm using novapak silica column an isocratic elution was employed using a mixture of ammonium dihydrogen phosphate buffer and methanol (21:79 v/v). The linearity range was 0.01-0.03 mg/ml of metformin. The accuracy was found to be 100.4%.[21]
12. P Madhusudhan *et al.*, (2015) developed and validated RP-HPLC method for simultaneous estimation of metformin and rosiglitazone in bulk and tablet dosage form. Isocratic chromatography was performed on a symmetry column (150mm x 4.6 mm, 5 µm, xTeera) with the mobile phase consisting of 70:30 % v/v (methanol: phosphate buffer) with the flow rate of 0.5 ml/min and detection at239 nm. The retention time for metformin and rosiglitazone was found to be 3.33 and 5.69 min respectively.[22]
13. Ashutosh *et al.*, (2015) developed and validated a stability indicating RP-HPLC method for simultaneous estimation of metformin and alogliptin in human plasma.

The drug was spiked in plasma and extracted by precipitation method. The extracted analyte was injected into xTeera column C_{18} (150 mm x 4.6 mm, 3.5 µm) temperature maintained at 25˚ C and effluent monitored at 235 nm. The mobile phase was sodium dihydrogen orthophosphate: ACN (70:30 v/v). The flow rate was maintained 1ml/min. The linear range from 300-700 µg/ml for metformin and 7.5-17.5 µg/ml for alogliptin, respectively.[23]

14. Chirag *et al.,* (2014) developed and validated UV spectrophotometric method for simultaneous estimation of metformin and alogliptin in bulk drugs and combined dosage form. Method A is simultaneous equation which is based on measurement of absorption at 277 nm and 232 nm. i.e, λ max of alogliptin and metformin respectively. Method B is Q absorbance ratio method which based on measurement of absorption at wavelength at 250 nm and 277 nm. i.e, iso-bestic point of alogliptin and metformin respectively. Linearity range of alogliptin 5-25 µg/ml and 1-10 µg/ml of metformin. The accuracy found within a range of 95-102 %.[24]
15. Dyade *et al.,* (2013) developed and validated uv visible spectrophotometric method for metformin and glimepiride. The dosage form contains 2 mg of glimepiride and 500 mg of metformin and the ratio of both drugs in the dosage form 1:250. Metformin freely soluble in water whereas glimepiride practically insoluble in water so first both the drugs are separated by solvent extraction method and individually determine by UV absorbance method. The linear range of metformin 8-40 µg/ml ad 5-30 µg/ml of glimepiride.[25]
16. Laxmi gowsami *et al.,* (2010) developed simultaneous estimation of metformin and pioglitazone by ultraviolet spectrophotometry. This method deals with the bilayer tablet dosage form without prior separation techniques. The methods employed are derivative spectrophotometric method and Q analysis. In the quantitative assay of two components by Q-analysis method, absorbances were measured at two wavelengths, one being iso-bestic point and other being the wavelength of maximum absorption of one of the two components. 247.5 nm was the iso-bestic point of both drugs and 231 nm was λmax of metformin. The linearity range of pioglitazone 0.5-4.5 µg/ml of pioglitazone and 16.5-150 µg/ml of metformin, respectively.[26]
17. B Amruta *et al*., (2012) developed and validated a simultaneous estimation of metformin and sitagliptin phosphate by UV. Two methods are developed method A is absorbance ratio method which is based on measurement absorption at maximum wavelength 266 nm and 232 nm for sitagliptin and metformin, respectively. Method B

area under curve in the wavelength range of 244 - 279 nm for sitagliptin and 222 - 240 nm for metformin, respectively. Linearity range was 25-225 µg/ml of sitagliptin and 2 - 12 µg/ml for metformin. The accuracy was found to be 99.64 and 98.9 % for sitagliptin and metformin, respectively.[27]

18. R Sejal *et al.,* (2012) developed and validated an analytical method for quantitative estimation of miglitol and metformin in combined dosage form. The developed method employs multicomponent spectroscopy using 300nm, 270 nm, 240 nm and 210 nm as wavelength for estimation. Miglitol and metformin were found to be linear in the concentration range of 0.2-1.2 µg/ml and 2-12 µg/ml, respectively. The accuracy was found to be within a range of 99.27 – 99.92 % and 99.29 – 99.97 % of metformin and miglitol, respectively.[28]
19. P Kabra *et al.,* (2011) developed a simultaneous estimation of Voglibose and Metformin by UV. The developed method employs multicomponent spectroscopic method using 325 nm, 285 nm, 245 nm and 205 nm. Voglibose was found to be absorbing prominently at 190 nm with smaller peak in the range of 325 nm to 245 nm while metformin absorbed at 232 nm. The content of analyte formulation was found to be 98-100.12%.[29]
20. K Darshana *et al.,* (2013) developed HPTLC method for simultaneous determination of Metformin and Sitagliptin in tablet dosage form. Chromatographic separation was performed on silica gel 60 F_{254} plates with buffer: water: glacial acetic acid (6:2:2 v/v/v) as mobile phase. The R_f value was 0.35 ± 0.01 for Metformin and 0.75 ± 0.01 for Sitagliptin. Detection was carried out at 227 nm.[30]
21. R Dhaneswar *et al.,* (2010) developed and validated HPTLC method for simultaneous estimation of Metformin, Atorvastatin and Glimepiride in bulk drugs and formulation. Chromatographic separation of the drug was performed on aluminum plates precoated with silica gel $60F_{254}$ as the stationary phase and the solvent system water: methanol: ammonium sulphate (1:1:4 v/v/v). Densitometric quantification at 237 nm. The R_f value was 0.37 ± 0.02, 0.59± 0.02, 0.75 ± 0.02 for Metformin, Atorvastatin and Glimepiride respectively. The linearity range was 200-700 ng/spot for Metformin, 600-2100 ng/spot for Atorvastatin and 600-2100 ng/spot for Glimepiride.[31]
22. Thomas *et al.,* (2011) developed a stability indicating HPTLC method for simultaneous estimation of nateglinide and metformin in pharmaceutical dosage form. Study was performed on pre-coated silica gel 60 F_{254} using chloroform: ethyl acetate: acetic acid (14:6:0.1 v/v/v) as a mobile phase. A TLC scanner set at 216 nm. The

correlation coefficients of calibration curve were found to be 0.996 and 0.995 in the concentration range of 200 – 2400 and 500 – 3000 ng/band for Nateglinide and Metformin, respectively.[32]

23. Veeresham *et al.,* (2010) developed and validated stability indicating HPTLC method for simultaneous estimation of Linagliptin and Metformin. Chromatographic separation was carried out a silica gel 60 F_{254} with acetone: methanol: chloroform: formic acid (3:1:5:1 v/v/v/v) as mobile phase followed by densitometrically analysed at 230 nm. The R_f value was 0.72 and 0.19 for Linagliptin and Metformin, respectively.[33]
24. Eman I.EI- kimary *et al.,* (2015) developed and validated HPTLC determination of three gliptins in binary mixtures with Metformin. The method was developed for the determination of Linagliptin, Saxagliptin (or) Vidagliptin in their mixture with metformin in pharmaceutical preparation using preferable green mobile phase system. Separation was carried out on Merck HPTLC silica gel 60 F_{254} plates using methanol: 0.5% ammonium sulphate (18:2 v/v) as mobile phase. Densitometrically analysed at 225 nm for Linagliptin, mixtures and 208 nm for both Saxagliptin and Vidagliptin mixtures. The linear regression range was 0.05-0.5 µg/band for Linagliptin and 0.2-2 µg/band for Saxagliptin and 5-40 µh/band for Vidagliptin.[34]
25. Vinyas *et al.,* (2016) developed and validated a RP-HPLC method for alogliptin. In this method ACN: phosphate buffer used as a mobile phase with the flow rate of 1 ml/min. the optimizing wavelength detection at 254 nm. The column used as Shisedio C_{18} (250mm x 4.6 mm, 5 µm). The linearity was observed in the range of 10-35 µg/ml and correlation co-efficient were 0.991.[35]
26. Komal Sharma *et al.,* (2015) developed and validated HPTLC method for estimation of Alogliptin in bulk drugs and tablet dosage form. Chromatographic separation was carried out on Merck HPLTLC aluminium sheets of silica gel 60 F_{254} using acetonitrile: 1% ammonium acetate in methanol (4.5:5.5 v/v) as mobile phase followed by densitometry analysis at 277nm. The reliability of the method was assessed by evaluation of linearity (500-5000 ng/spot) for Alogliptin. The accuracy of the method was found to be in the range of 98-102 %. Due to non-availability of the product, 400 mg tablets were punched containing 34 mg of Alogliptin.[36]
27. Supriya *et al.,* (2016) developed and validated UV spectrophotometric and RP-HPLC-PDA methods for the estimation of Alogliptin. UV spectrophotometric method was performed using UV/Vis double beam spectrophotometer with a spectral bandwidth

of 1nm and 1 cm matched quartz cells. The maximum absorbance of Alogliptin was observed at 276 nm using methanol as a solvent. RP-HPLC method was carried out on a Unisol RP C_{18} column (150 mm x 4.6 mm, 4.6 µm) with a mobile phase composed of methanol and 10 mM ammonium acetate in the ratio of 80:20 v/v with a flow rate of 0.8 ml/min. The linearity of the method was found to be in the range of 5-35 µg/ml (uv) and 20-100 µg/ml (RP-HPLC) and the correlation values was 0.999 for both the methods.[37]

28. Sunilkumar *et al.,* (2016) developed a visible spectrophotometric method for quantification of alogliptin using utility of picric acid and 2, 4 dinitrophenol as chromogenic reagent. Two simple and sensitive visible spectrometric method (A & B) were developed for the estimation of alogliptin in bulk and its tablet dosage form. The methods use the reaction of alogliptin with picric acid (method A) or 2, 4 dinitrophenol (method B) in the chloroform medium. The complex alogliptin with picric acid (method A) or 2, 4 dinitrophenol (method B) showed λmax at 415 nm and 430 nm respectively. The different conditions affecting the formation and stability of the complexes were optimized. The methods were validated statistically according to ICH. The calibration curve is linear in the range of 10-60µg/ml and 10-50µg/ml for methods A and methods B, respectively.[38]
29. Venugopala rao *et al.,* (2014) developed and validated a chiral HPLC method for the enantiomeric purity of alogliptin benzoate. An isocratic chiral stationary phase was used for the quantitation of (S) - isomer in alogliptin. Separation was achieved with a Lux cellulose 3 (250 x 4.6 mm, 5 µm) column. The ratios of ethanol and diethyl amine in the mobile phase were optimized to obtain the best separation. UV detection was performed at 230 nm. The described method is linear over a range of LOQ – 1.5µg/ml of (S)- isomer.[39]
30. Ida Fejos *et al.,* (2014) developed a separation of alogliptin enantiomers in cyclodextrin modified capillary electrophoresis. Preliminary screening of the native CDs and their ten derivatives revealed that sulfopropylated-γ-CD, sulfopropylated-β-CD and sulfopropylated-γ-CD, sulfobutyl-ether-β-CD (SBE-β-CD) and sulfobutyl-ether-γ-CD enabled enantioresolution. Furthermore, cavity size dependent enantiomer migration order reversal was observed between γ- and β-CD derivatives. To improve enantioseparation, buffer composition and pH, CD concentration, applied voltage, temperature, and injection parameters were optimized for the Alo/ SBE-β-CD system, yielding a resolution of 8.34. RSD percentage of the resolution value, migration times,

and corrected peak areas were below 3 and 5% during testing repeatability and intermediate precision. LOD and LOQ values were found to be 2 and 6 µg/mL, respectively, for each enantiomer. Calibration lines ranging from 6 to 250 µg/mL were constructed with $r^2 > 0.9997$. Robustness could be successfully verified by using the Plackett–Burman statistical experimental design. The optimized system containing 5 mM SBE-β-CD in a 25 mM acetate buffer at pH 4.75 was found promising to detect 0.1% distomer in the presence of the API.[40]

31. I Ramzia *et al.,* (2012) developed a liquid chromatographic determination of alogliptin in bulk and pharmaceutical preparation. This method developed based on isocratic elution using a mobile phase containing potassium dihydrogen orthophosphate buffer: acetonitrile (20:80 v/v) at a flow rate of 1 ml/min with UV detection at 215 nm. Chromatographic separation was achieved on a symmetry cyanide column (150 mm x 4.6 mm, 5µm). Linearity, accuracy and precision were found to be acceptable range of 5-160 µg/ml for alogliptin in bulk.[41]
32. M Anusha *et al.,* (2014) develop and validate RP-HPLC method for Pioglitazone and alogliptin in combined tablet dosage form. Determination of alogliptin and Pioglitazone using mobile phase (mixture phosphate buffer and acetonitrile in the ratio of 35:65) as the solvent. The proposed method involves the measurement of retention time at selected wavelength. 268 nm was selected as the analytical wavelength. The retention time of alogliptin and Pioglitazone was found to be 3.113 and 5.234 respectively. The linearity of the proposed method was investigated in the range of 2.5-15 µg/ml. for alogliptin and 3-18 µg/ml for Pioglitazone respectively.[42]
33. Komal Sharma *et al.,* (2015) developed and validated HPTLC method for simultaneous determination of alogliptin and Pioglitazone in bulk drugs and combined dosage form. Chromatographic separation was carried out using Merck HPTLC silica gel 60 F_{254} plates using acetonitrile: 1% ammonium acetate in methanol (4.5:5.5 v/v) as mobile phase followed by densitometry analysis at 254 nm. The reliability of the method was assessed by evaluation of linearity (500-3000 ng/spot for alogliptin as well as Pioglitazone).The accuracy of the method was found to be within a range of 98-102%.[43]
34. Anees *et al.,* (2015) developed a simultaneous estimation of alogliptin and metformin from its tablet dosage form by area under curve and multicomponent uv spectrophotometric method. Two simple, accurate and precise UV methods were developed for the estimation of alogliptin and metformin in tablet dosage form.

Method A is area under curve spectrophotometry and in this the wavelength range selected for quantitation are 215- 240 nm for alogliptin and 265-293 nm for metformin. Method B is multicomponent mode wavelength selected for quantitation method were metformin and alogliptin therefore for 284nm (λ max of metformin) and 274 (λ max of alogliptin) for the analysis. In both the methods linearity for detector response was observed in the concentration range of 10-50 µg/ml for MET and ALO respectively. The results of tablet analysis for area under curve was found to be 99.90 ±0.151 for ALO and 99.62 ±0.220 for MET and result obtained for multicomponent was 99.73 ± 0.306 for ALO and 99.30 ±0.224 for MET.[44]

35. Dimal e*t al.*,(2017) developed a estimation of Pioglitazone Hydrochloride and Alogliptin Benzoate in Combination by Second Order Derivative Spectrophotometry method. A simple and accurate method for analysis of Pioglitazone hydrochloride and alogliptin benzoate in their combination was developed using second order derivative spectrophotometry. Pioglitazone and alogliptin were quantified using second order derivative responses at 267 nm and 278 nm prepared in methanol. The calibration curves were found to be linear in the concentration range of 10-30 µg/ml for both Pioglitazone and alogliptin.[45]
36. K Swathi *et al.,* (2015) developed the simultaneous estimation of metformin and alogliptin by using RP-HPLC. Chromatographic separations were done by using the column X Bridge RP C_{18} (4.6 mm x 50 mm). Methanol: Acetonitrile: Phosphate buffer (pH 7) in the ratio of 40:20:40. The flow rate was maintained at 1 ml/min. both the drugs are dissolved in the mobile phase. The UV spectrum was taken for selection of wavelength. The isobestic point was taken as detection wavelength. The linearity study of metformin and alogliptin was found in the concentration range of 50 µg to 250 µg and 5µg to 25 µg.[46]
37. S.Mowaka *et al.,* (2017) developed a comparative study between UHPLC-UV and UPLC-MS/MS methods for determination of alogliptin and metformin in their pharmaceutical combination. Concerning method A, separation was achieved by Hypersil gold (50mm x2.1 mm, 1.9 µm) column, using acetonitrile and 0.2%formic acid aqueous solution as the mobile phase with a gradient elution. Electrospray ionization source was operated in a positive ion mode. Selected reaction monitoring mode on a triple quadropole mass spectrometer was used do quantify the drugs utilizing the transitions of 340.33- 116.32 (*m/z*) and 130.12- 71.32 (*m/z*) for alogliptin and metformin, respectively. Concerning method B, it was achieved on a Symmetry

C_{18} column (100mm x 2.1 mm, 2.2 µm) applying an isocratic elution based on methanol: water in the ratio of (10:90 v/v) at pH 3 as mobile phase. The photodiode array detector was operated at 210 nm. Method A showed the good linearity over the concentration range of 5-400 ng/ml and 20-2000 ng/ml for alogliptin and metformin, respectively, while method B showed satisfactory results using ranges of 0.25-8 µg/ml and 5-50µg/ml for alogliptin and metformin, respectively.[47]

38. G Hemavathi *et al.*, (2017) developed a sensitive LC-MS/MS method for simultaneous determination of alogliptin and voglibose in human plasma. A highly sophisticated and sensitive LC-MS/MS method has been developed and validated for the alogliptin and voglibose simultaneous determination in human plasma. Alogliptin D3 and miglitol were used as IS. Chromatography conditions included an isocratic mobile phase consisting of 5 mM ammonium formate: acetonitrile in the ratio of 20:50 v/v. the column used was Welchrom XB C_{18}, with specification of 50 x 4.6 mm, 5µm, at a flow rate of 0.70 ml/min. The retention time of alogliptin, voglibose, alogliptin D3 and miglitol occurred at 1.03, 0.8, 0.8 and 0.81 min respectively and the total chromatographic run time was 3.0 min. Alogliptin and voglibose achieved a linear regression in human plasma at 5.09-509 ng/ml and 2.03-203 ng/ml respectively.[48]

AIM AND PLAN OF WORK

AIM

Analytical methods are intended to establish the identity, purity, physical characteristics and potency of the drugs. Methods may also support safety and characterization studies or evaluation of drug performance.

From the viewpoints mentioned above, the present work was undertaken to develop and validate an analytical methods for the estimation of metformin and alogliptin in pharmaceutical dosage form and from plasma.

PLAN OF WORK

Literature survey revealed that analytical methods have been reported for the estimation of the metformin and alogliptin individually and in combination with other drugs. Hence, the present work aims to develop and validate a spectroscopic and chromatographic method for estimation of the metformin and alogliptin from pharmaceutical dosage form and from plasma in the following stages;

- Selection, optimization of chromatographic parameters
- Validation of proposed methods
- Application of proposed methods to formulation

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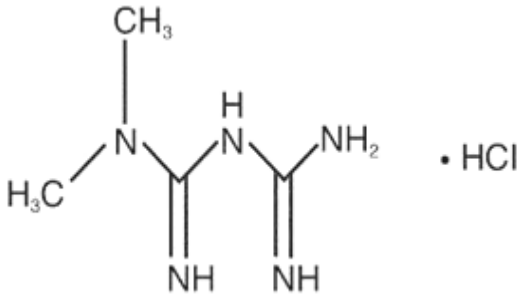
DRUG PROFILE

METFORMIN

Chemical structure :



Molecular weight : 129.16364 g/mol

IUPAC name : 3-(diaminomethylidene)-1,1-dimethylguanidine

Empirical formula : $C_4H_{11}N_5$

Pharmacopeial status : official in IP,BP, USP

Description : white crystalline powder

Solubility : Freely soluble in water and practically insoluble in acetone, ether and chloroform.

Contra indications : kidney disorders, lung diseases, liver diseases.

Pharmacological action

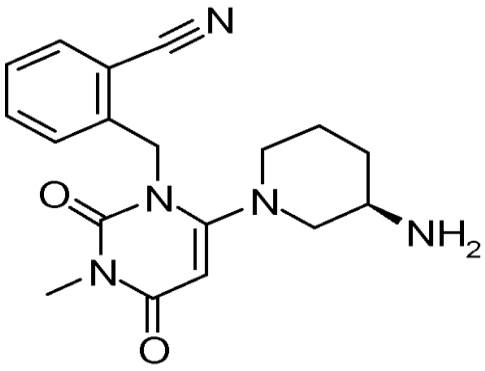
Oral hypoglycemic agent. It is used in the first line therapy in the treatment of type –ii diabetes mellitus. It decreases blood glucose levels by decreasing hepatic glucose production and improving insulin sensitivity by increasing peripheral glucose uptake and utilization.

Adverse drug reaction : gastrointestinal irritation including diarrhoea, nausea, vomiting.

Storage : store at room temperature [49]

ALOGLIPTIN

Chemical structure :



Molecular weight **:** 339.39 g/mol

IUPAC name **:** 2-({6-[(3*r*)-3-aminopiperidin-1-yl]-3-methyl-2,4-dioxo-3, 4-dihydropyrimidin-ethyl) benzonitrile

Empirical formula **:** $C_{18}H_{21}N_5O_2$

Pharmacopeial status **:** official in USP

Description **:** colourless fine powder

Solubility **:** soluble in methanol, sparingly soluble in water

Indications **:** Indicated as an adjunct diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus.

Pharmacological action

Novel hypoglycemic drug belongs to dipeptidyl peptidase-4 inhibitor group, which normally degrades the incretins glucose dependant insulin tropic polypeptide (git) and glucagon like peptide.

Adverse drug reaction **:** hypoglycaemia, joint pain.

Storage **:** **s**tore in cool temperature.

Presentation **:** film coated tablet.

Trade name **: Kazano [Metformin 500mg, Alogliptin 12.5mg]** [50, 51]

MATERIALS AND INSTRUMENTS

Drug sample

- Metformin HCl was received from Cadila pharmaceutical, Ahmedabad, Gujarat.
- Alogliptin was kindly supplied by Vivan Life sciences, Thane, Maharashtra.
- The formulation KAZANO® (Metformin and Alogliptin) 500-12.5mg tablets (Takeda, Canada) were purchased from Pharmasave pharmacy, Canada.

Chemicals and solvents used

- Methanol HPLC grade - Himedia laboratory Pvt Ltd, Mumbai.
- Water for HPLC – RFCL Ltd, Delhi.
- Acetonitrile HPLC grade Sigma-Aldrich chemicals Pvt Ltd, Bangalore.
- Ammonium solution 30% Loba chem., Mumbai.
- Potassium dihydrogen orthophosphate Himedia laboratory Pvt Ltd, Mumbai.
- Ammonium sulphate (0.5 % v/v) – Hi-pure fine chem industries, Chennai.
- Chloroform – Avantor performance materials India Ltd, Maharashtra.

Instruments used `

HPLC System	Shimadzu
HPLC Software	Lab solution
Pump	Shimadzu LC-10ATVP
Photodiode array detector	Shimadzu (SPD- M10AVP)
Column	Phenomenex C_{18} (250x4.6mm ID, 5µm)
UV double beam spectrophotometer	UV- 1650 PC Shimadzu, Japan
HPTLC system	CAMAG (winCATS)
pH meter	LABINDIA, New Delhi
Sonicator	Ultrasonic
Centrifuge	Eppendrof 5415
Analytical balance	AND HR-200

DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF ALOGLIPTIN AND METFORMIN IN COMBINED DOSAGE FORM BY UV METHOD

SELECTION OF SOLVENT

Solution of metformin hydrochloride (40 µg/ml) and alogliptin (1 µg/ml) were prepared in Methanol, 0.05N Sodium hydroxide and Acetonitrile. UV spectrum of the solution recorded and overlain spectra of metformin and alogliptin in the solutions shown in the **Fig.1 to 3.**

SELECTION OF WAVELENGTH

Absorbances of both the drugs were higher and gave good sharp peak in methanol, so it was decided to prepare drug solution in methanol for further studies. The λ_{max} of metformin and alogliptin were found to be 237 nm and 225 nm respectively.

ANALYSIS OF FORMULATION

PREPARATION OF STANDARD SOLUTION

Stock solutions of metformin (400 µg/ml) and alogliptin (10 µg/ml) are prepared in methanol. A solution of metformin (40 µg/ml) and alogliptin (1 µg/ml) and mixture of metformin and alogliptin (40 µg/ml and 1 µg/ml) were prepared in methanol. The solutions were scan in UV region and the overlain spectra as shown in **Fig. 4.** From overlain spectra, wavelength 237nm and 225nm were selected. The different concentration of metformin (2 µg/ml to 10 µg/ml) and alogliptin (0.05 µg/ml to 0.25µg/ml) and mixture of metformin and alogliptin (2.05 µg/ml to10.25 µg/ml) were prepared from respective stock solution and scan in UV region and spectrum shown in **Fig 5, 6.**

The absorbances were noted at selected wavelengths, **Table 1 and 2.**

PREPARATION OF SAMPLE SOLUTION

20 tablets each containing 500 mg of metformin and 12.5 mg of alogliptin were weighed and quantity equivalent to 40 mg of metformin and 1 mg of alogliptin were dissolved in Methanol. Sonicated for 20 mins and make upto the mark by using same. The solution was filtered and further diluted to get a concentration 40 µg/ml and 1 µg/ml of metformin and alogliptin respectively. The UV spectrum shown in **Fig. 11.**

Absorbances were noted at 237 nm and 225 nm and amount of metformin and alogliptin were calculated using simultaneous equation method, formula is given below.

$$c_x = \left(\frac{A_2 a_{y1} - A_1 a_{y2}}{a_{x2} a_{y1} - a_{x1} a_{y2}} \right)$$

And

$$c_y = \left(\frac{A_1 a_{x2} - A_2 a_{x1}}{a_{x2} a_{y1} - a_{x1} a_{y2}} \right)$$

Where,

A_1 and A_2 are absorbances of formulation at 237 and 225 respectively,

C_x and C_y are the concentration of metformin and alogliptin respectively,

ax_1 and $ax_2$are absorptivities of metformin at 237 and 225 respectively,

ay_1 and ay_2 are absorptivities of alogliptin at 237 and 225 respectively,

% Label claim and estimated amount had shown in **Table No 3.**

VALIDATION

LINEARITY:

1. Metformin

   Metformin was found to be linear at the concentration range of 2-10 µg/ml. The absorbance of this solution was noted at the wavelength 237nm and 225nm and calibration curve were plotted using concentration Vs absorbance. **Fig.7and 8.**

   At wavelength 237 nm- slope, intercept and correlation coefficient values were found to be 0.10125, 0.01810 and 0.99936.

   At wavelength 225 nm- slope, intercept and correlation coefficient values were found to be 0.0630, 0.05180 and 0.99963.

2. Alogliptin

   Alogliptin was found to be linear at the concentration range of 0.05-0.25 µg/ml. The absorbance of this solution was noted at the wavelength 237nm and 225 nm and calibration curve were plotted using concentration Vs absorbance. **Fig. 9 and 10.**

   At wavelength 237 nm- slope, intercept and correlation coefficient values were found to be 0.13000, 0.00030 and 0.933.

   At wavelength 225 nm- slope, intercept and correlation coefficient values were found to be 0.10000, 0.0232 and 0.9968.

ACCURACY

To study the reliability, suitability and accuracy of the method recovery experiments were carried out. To the formulation equivalent to 40 mg of metformin and 1 mg of alogliptin at the level of 80 % and 100 % pure metformin and alogliptin were added and suitably diluted. The contents were determined from respective chromatogram and table shown in **Table 4.**

PRECISION

Precision of the method was demonstrated by,

- Intraday precision
- Interday precision

1. Intraday precision

Intraday precision was found out by carrying out analysis of standard drug solution at three different concentrations in the linearity range for three times on the same day and %RSD was calculated.

2. Interday precision

Interday precision was found out by carrying out analysis of standard drug solution at three different concentrations in the linearity range for three day over a period of one week and %RSD was calculated.

STABILITY

The sample solution was subjected to stability studies under room condition. Stability was studied looking for any change in absorbance and peak shape when compared to UV spectra of freshly prepared solution. The solution store under room temperature was stable upto 3 hours.

OVERLAIN SPECTRA OF METFORMIN AND ALOGLIPTIN IN VARIOUS SOLVENTS

Fig.1. Overlain spectra in Methanol

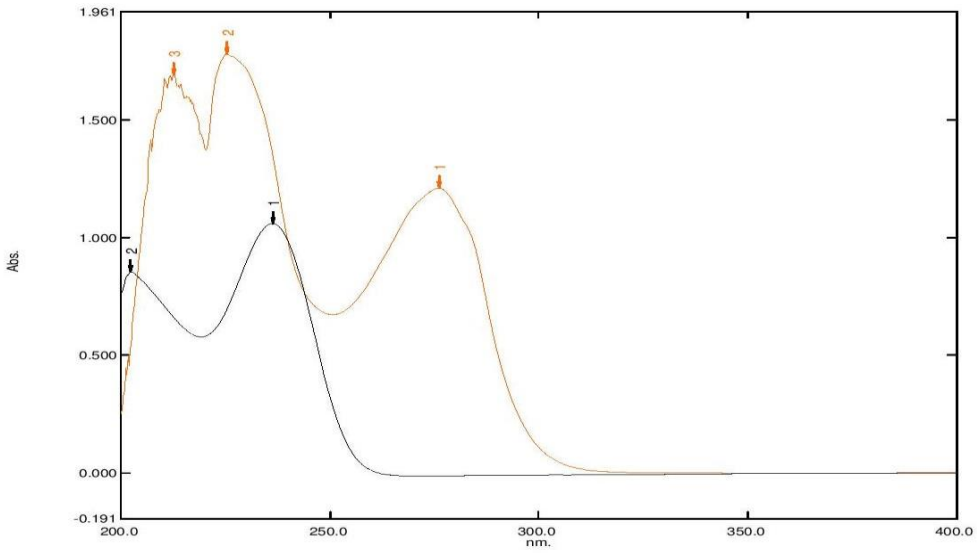


Fig. 2. Overlain spectra in 0.05N sodium hydroxide

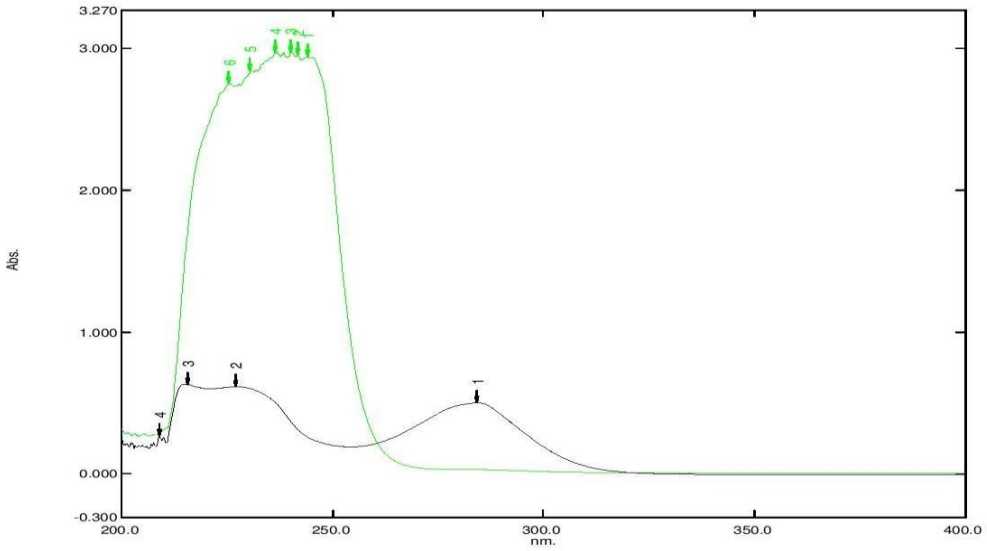


Fig.3. Overlain spectra in Acetonitrile

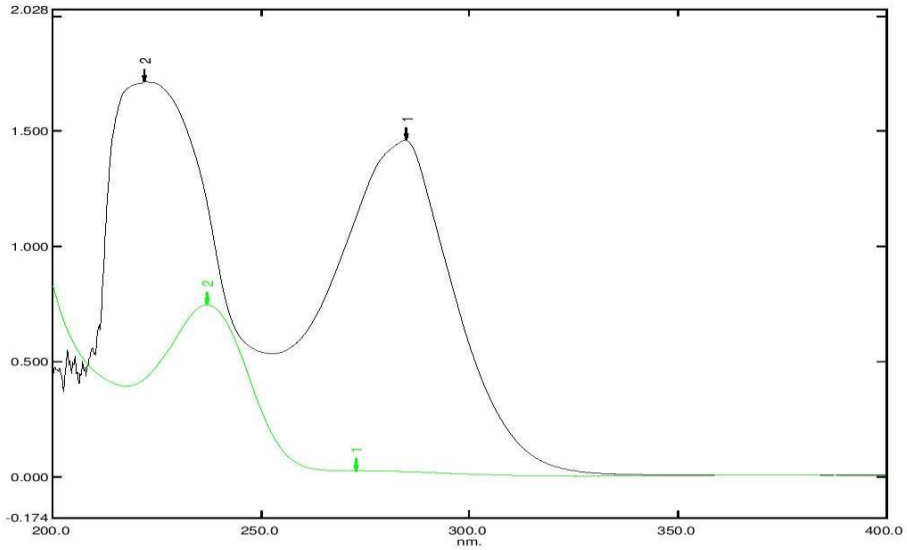


Fig. 4. Overlain spectra of Metformin, Alogliptin and Mixture

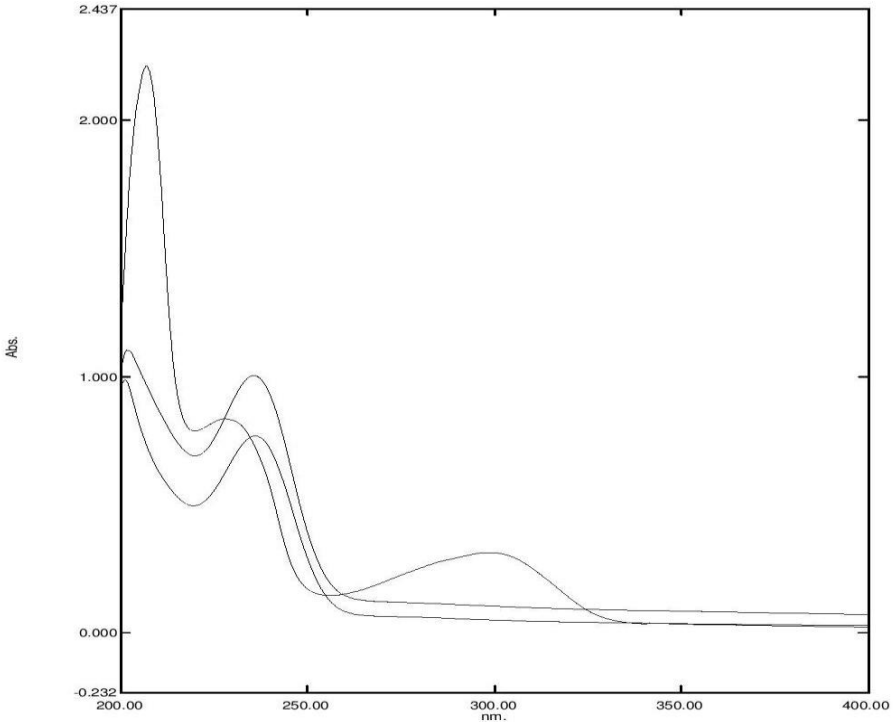


Fig. 5. Overlain spectra of Metformin (2-10 µg/ml)

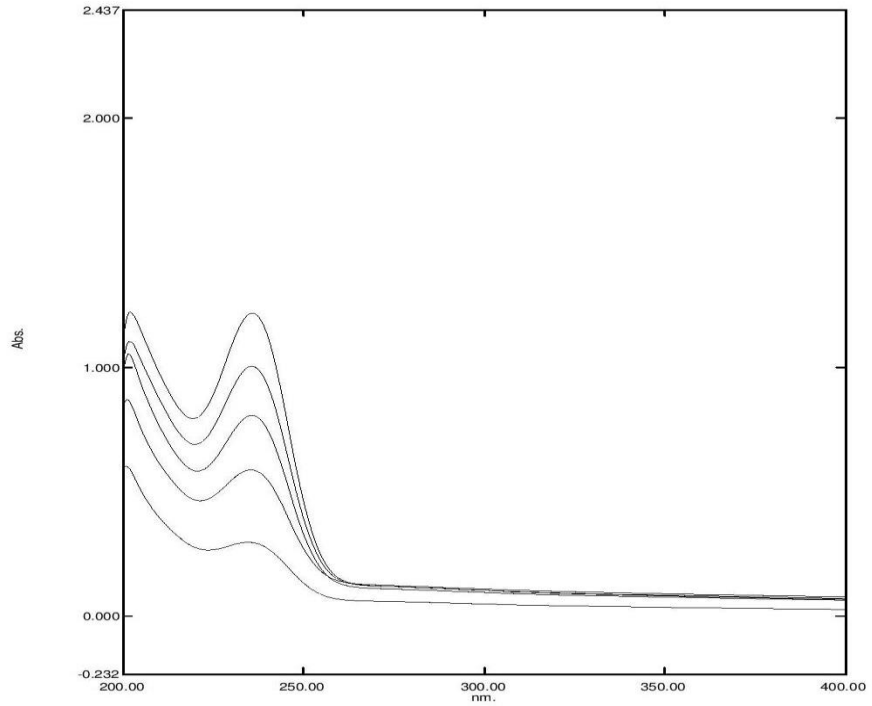


Fig. 6. Overlain spectra of Alogliptin (0.05-0.25 µg/ml)

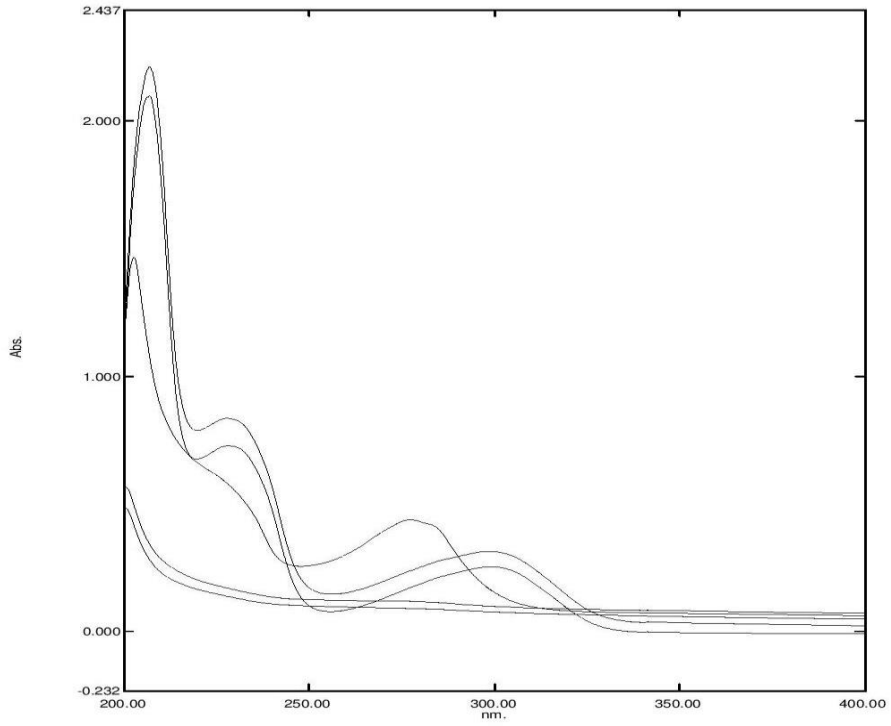


Table No.1 Absorbance of metformin at selected wavelength.

S. No	Concentration (µg/ml)	Absorbance at 237nm	Absorbance at 225nm
1	2	0.217	0.181
2	4	0.420	0.298
3	6	0.640	0.433
4	8	0.823	0.554
5	10	1.028	0.683

Table No.2 Absorbance of alogliptin at selected wavelength.

S. No	Concentration (µg/ml)	Absorbance at 237nm	Absorbance at 225nm
1	0.05	0.009	0.028
2	0.1	0.010	0.033
3	0.15	0.019	0.039
4	0.2	0.023	0.043
5	0.25	0.035	0.048

Fig.7. Calibration graph of Metformin at 237nm

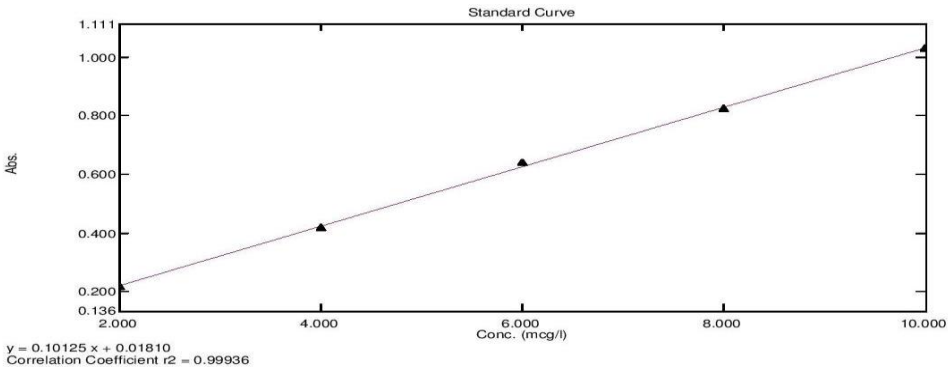


Fig.8. Calibration graph of Metformin at 225nm

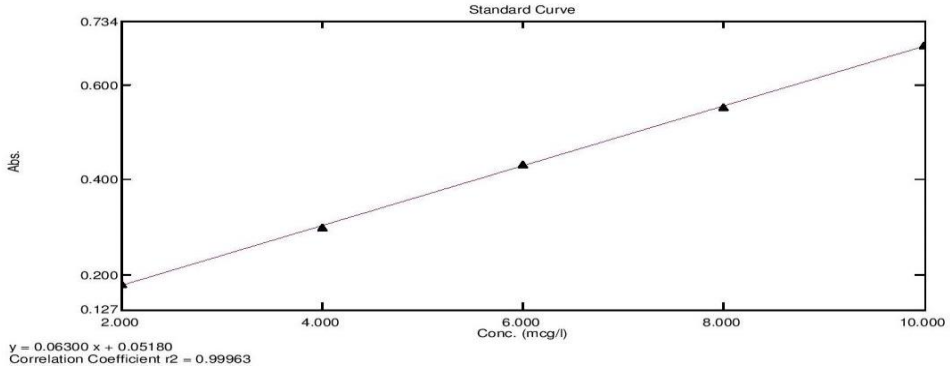


Fig.9. Calibration graph of Alogliptin at 237nm

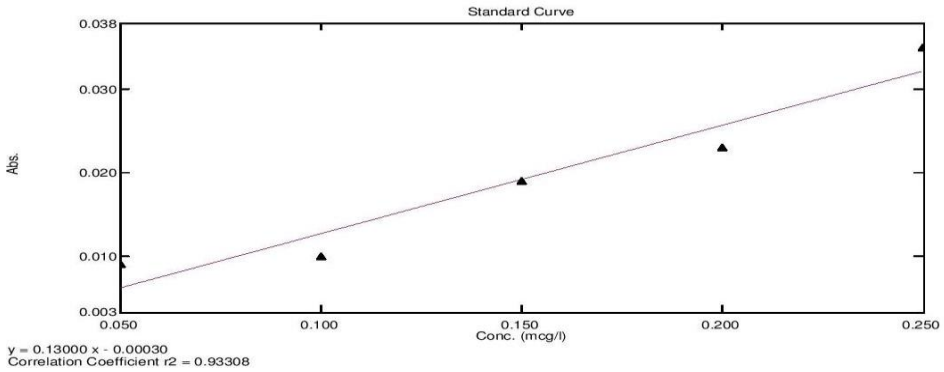


Fig.10. Calibration graph of Alogliptin at 225nm

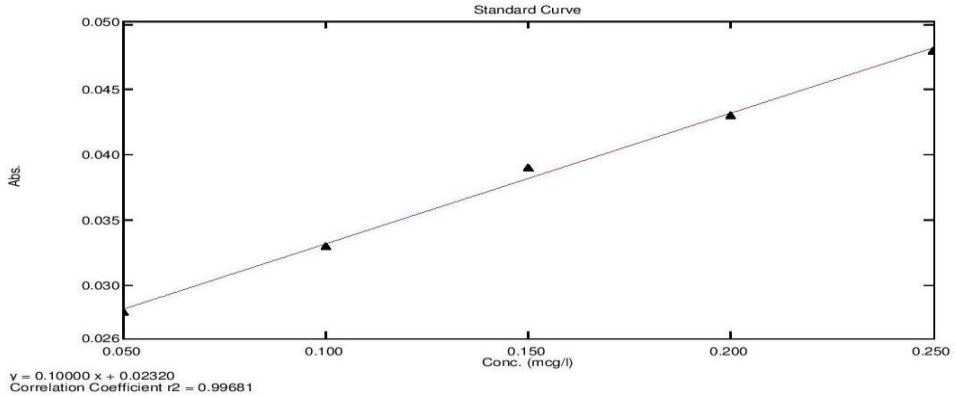


Table No.3 Analysis of formulation

S.No	Drug	Amount(mg)		%Label claim	%RSD*
		Labeled	Found		
1	MET	500	498	99.6	0.32
2	ALO	12.5	12	96	0.85

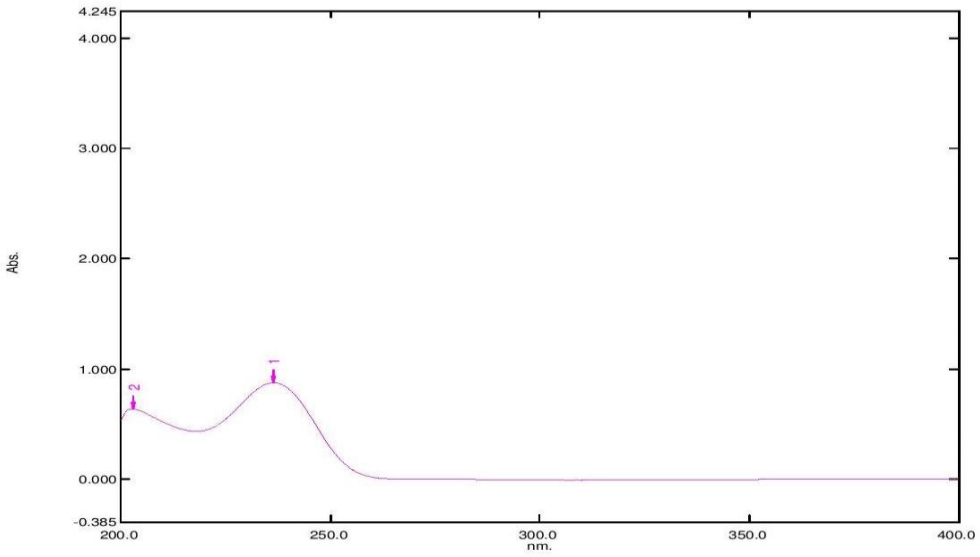
*RSD of five observations

Table No.4 Recovery studies

S.No	Level	%Recovery		%RSD*	
		MET	ALO	MET	ALO
1	80%	99.82	98.60	0.201	0.260
2	120%	98.80	101.50	0.106	0.424

*RSD of five observations

Fig.11. Spectra of formulation



DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR SIMULTANEOUS DETERMINATION OF ALOGLIPTIN AND METFORMIN IN FIXED DOSE COMBINATION TABLET

SELECTION OF SOLVENT

Ideal properties of solvents are listed below:

- Drug should be freely soluble in the solvent used
- Drug should show stability in solvents used
- Solvent should be volatile

Accordingly methanol was selected as solvent for further studies.

SELECTION OF WAVELENGTH

The maximum wavelength absorption of drug at 254 nm which was selected as the detection wavelength.

SELECTION OF STATIONARY PHASE

Different plates are available with the different size, which should be selected based on the analyzing compound. HPTLC Silica gel 60 F_{254} HPTLC (10*10 or 10*20) plates are used as a stationary phase for the studies.

MOBILE PHASE SELECTION AND OPTIMIZATION

Mobile phase for HPTLC method is selected and optimized on the basis of analyst's own experience, literature report of similar studies and traditional trial and error method. A solvent system that would give dense compact spots and good separation from the application position and solvent front was to be selected.

Initially different solvent systems like

1. Chloroform : Methanol (4:6)
2. Ethyl acetate : Formic acid : Chloroform (2:3:5)
3. Chloroform : Methanol : Ammonia (5:3:2)
4. n - butanol : Ethanol : Ammonia (4:4:2)

5. Acetone : Methanol : Toluene : Formic acid (4:3:2:1)
6. Acetonitrile : Chloroform (4:6)
7. Methanol : Ammonia : Acetone : Formic acid (3:2:4:1)
8. Chloroform : Ammonia : Acetone : Formic acid (3:2:4:1)
9. Methanol : Ammonium sulphate (0.5%) : Propanol : Ammonia (3:4:2:1)
10. Acetonitrile : Methanol : Ammonium acetate (1%) : Ammonium sulphate (0.5%) (3:3:2:2)
11. Chloroform : Methanol : 0.5% Ammonium sulphate (4:4:2)

Among these systems, Chloroform: Methanol: 0.5% Ammonium sulphate (4:4:2) was selected because in this system good compact and dense spots were obtained.

Separation using Chloroform: Methanol: 0.5 % Ammonium sulphate

Different ratios of the Chloroform: Methanol: 0.5 % Ammonium sulphate as tried and from a ratio of (4:4:2 v/v/v) was selected because it gave good separation from the application position and solvent front.

FIXED EXPERIMENTAL CONDITION

Stationary phase	:	pre – coated silica gel 60 F_{254} on aluminium sheets
Mobile phase	:	Chloroform: Methanol: Ammonium sulphate (0.5%) (4:4:2)
Chamber saturation	:	20 min
Migration distance	:	7 cm
Band width	:	8mm
Slit dimension	:	6 x 0.3 mm
Source of radiation	:	Deuterium lamp
Wavelength of scanning	:	254 nm
R_f values		
Metformin	:	0.44 ± 0.02
Alogliptin	:	0.66 ± 0.02

ANALYSIS OF FORMULATION

PREPARATION OF STANDARD SOLUTION

Accurately weighed 10 mg quantity of Alogliptin and 400 mg of Metformin were taken and transferred into a 10 ml clean volumetric flask. The drugs were dissolved in methanol and made up to the volume methanol to obtain 1000 µg/ml of Alogliptin and 40000 µg/ml of Metformin (stock solutions). Aliquots of standard solutions were applied in the concentration range of 100-500 ng/spot of Alogliptin and 4000-20000 ng/spot of Metformin by applying 0.1 -0.5 µl prepared standard solutions, which were used for calibration purpose.

PREPARATION OF SAMPLE SOLUTION

For analysis of tablet dosage form, twenty tablets, each containing 12.5 mg of Alogliptin and 500mg of Metformin, were weighed and their average weight was calculated. The tablets were finely powdered and powder equivalent to 10 mg Alogliptin and 400mg Metformin were accurately weighed and dissolved in 10 mL of methanol. The solution was sonicated for 30 min, filtered through the Whatman No. 41 filter paper and the residue was washed with methanol. This solution was further diluted with methanol to get the same concentration as that of the final standard solution. The chromatogram was shown in **Fig.15.**

CHROMATOGRAM DEVELOPMENT

Alogliptin and Metformin reference standard solutions were prepared using methanol as solvent. Solutions of 0.1-0.5 µL were applied to the HPTLC plates as spot bands of 6 mm using LINOMAT V. Application positions were at least 15 mm from the sides and 10 mm from the bottom of the plates. Mobile phase components were mixed prior to use and the development chamber was left for saturation with mobile phase vapor for 20 mins before each run. Development of the plate was carried out by the ascending technique to a migration distance of 7 cm. Then the plates were dried on a hot plate. All the analyses were carried out in a laboratory with temperature control (20–24ºC).

DETECTION

Densitometry scanning was done in absorbance mode at 254 nm using a deuterium lamp. The slit dimensions were set at 6 mm-0.30 mm, the scanning speed of 10 mm/s, and the data resolution at100 µm/step. Single wavelength detection was performed since the main components were only analyzed.

VALIDATION OF THE METHOD

The developed method was validated as per the International Conference on Harmonization (ICH) guidelines with respect to linearity and range, specificity, precision, accuracy, limit of detection and limit of quantification.

LINEARITY AND RANGE

A stock standard solution was prepared for both Alogliptin and Metformin they were serially diluted to yield five standard solutions. A volume of 0.1-0.5 µL of each solution was applied to the HPTLC plate to deliver 100, 200,300, 400 and 500 ng/spot of Alogliptin, 4000, 8000, 12000, 16000 and 20000 ng of Metformin /spot. The calibration plots revealed good linear relationships between area and concentration over the range of 100-500 ng/spot for Alogliptin and 4000-20000 ng/spot for Metformin. The standard curves for Alogliptin and Metformin are shown in **Fig.12** and **Fig.13,** respectively and data for both Alogliptin and Metformin is presented in **Table 9 and 10**.

ACCURACY

Accuracy of the method was determined by replicates (n=3) analysis, carried out using three solutions prepared by standard addition of pure active pharmaceutical ingredient at three different concentration levels 80%,100% and 120%. Accuracy was calculated by comparing the difference between the spiked value (theoretical value) and that actual found value. Results are presented in the tem of %recovery of the active pharmaceutical ingredient and data for both Alogliptin and metformin is presented in **Table 5.**

LIMITS OF DETECTION AND QUANTIFICATION

The limit of detection was found to be 2 ng/spot and 40 ng/spot for Alogliptin and Metformin, respectively. The limit of quantification was found to be 6 ng/spot and 130 ng/spot for Alogliptin and Metformin, respectively, which was lower than that reported earlier.

PRECISION

Precision of the method was demonstrated by

a. Intraday precision
b. Interday precision
c. Repeatability

a) Intraday Precision

Intra-day precision was found out by carrying out the analysis of the standard drug at three different concentrations of 100,200and 300 ng for Alogliptin, 4000, 8000 and 12000 ng for Metformin were selected from linearity range. Intraday analysis was carried on same day in three replicates. Each concentration was applied in duplicate and %RSD was calculated and the results shown in **Table 6**.

b) Interday Precision

Inter-day precision was found out by carrying out the analysis of the standard drug at three different concentration of 100,200and 300 ng for Alogliptin, 4000, 8000 and 12000 ng for Metformin were selected from linearity range. Interday analysis was carried on three different days in three replicates. Each concentration was applied in duplicate and %RSD was calculated and the results shown in **Table 7.**

c) Repeatability

Repeatability was determined by applying 0.4 µL of standard solution containing 400 ng/spot of Alogliptin and 16000 ng/spot of Metformin in six replicates and respective areas were calculated. The %RSD was calculated and the results are shown in **Table 8.**

SPECIFICITY

The chromatogram of the solution, which was not spiked with Alogliptin and Metformin, did not show any spot, while the chromatogram of the solution of the tablet matrix spiked with Alogliptin and Metformin showed clear, compact and well separated peaks of Alogliptin and Metformin **Fig.14.** Moreover, from Fig.14, it can be seen that no other peaks were eluted besides the two active compounds. The method was therefore considered to be specific. Different concentration of metformin and Alogliptin are analysed and the chromatogram was shown in **Fig.16 to 20.** The UV spectrum of metformin and Alogliptin and overlay are

analysed through winCATS software and spectrum shown in **Fig.21 to 23.** The 3D chromatogram of metformin and alogliptin shown in **Fig .24.**

ROBUSTNESS

The robustness of an analytical method evaluates the method capacity to remain unaffected by minor but purposeful variations in the method parameter and provides an indication of its reliability during normal usage. Robustness of the developed method was evaluated by the analysis of sample solution after making small changes in the mobile phase volume and mobile phase saturation time. The low value of % RSD shows that the method is robust and that a slight change in mobile phase volume and mobile phase saturation time does not affect the results

Table.5: Accuracy for Alogliptin and Metformin

Level	% Recovery		% RSD	
	ALG	MET	ALG	MET
80 %	100	102.5	0.349	0.484
100 %	98.87	100.24	0.375	0.440
120 %	101.75	100.30	0.472	0.308

Table.6: Intraday precision data for Alogliptin and Metformin (n=3)

Level	Concentration (ng/ml)		Peak area		% RSD	
	ALG	MET	ALG	MET	ALG	MET
I	200	8000	585.3	12349	0.43	1.05
			582.1	12566		
			580.3	12329		
II	300	12000	600.2	12449	0.61	1.03
			598	12226.5		
			593	12229.5		
III	400	16000	500.8	12925.4	1.04	0.88
			496.2	12966.6		
			490.5	12751.7		

RSD Relative standard deviation

Table.7: Interday precision data for Alogliptin and Metformin (n=3)

Day	Concentration (ng/ml)		Peak area		%RSD	
	ALG	MET	ALG	MET	ALG	MET
1	200	8000	550	7142.5	0.66	0.91
2			553	7033.2		
3			546.2	7028.1		
1	300	12000	600.5	10091.5	0.35	0.59
2			596.3	10188		
3			598	10076		
1	400	16000	750.3	15996.3	0.70	0.30
2			746	15953.2		
3			756.5	15900.2		

RSD Relative standard deviation

Table.8: Repeatability data for Alogliptin and Metformin (n=6)

Concentration	Injection	Peak area		%RSD	
		ALG	MET	ALG	MET
Alogliptin(0.4 µg) Metformin (16 µg)	1	1458.5	12745.1	0.82	1.03
	2	1443.0	12470.1		
	3	1456.4	12365.4		
	4	1435.6	12569.5		
	5	1436.2	12458.9		
	6	1428.1	12487.8		

RSD Relative standard deviation

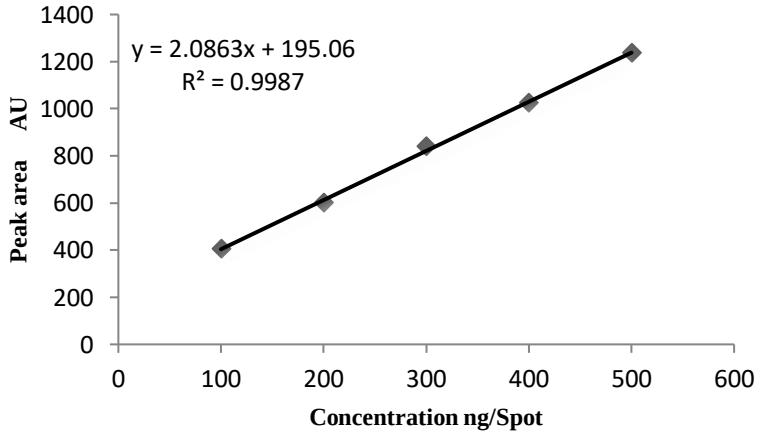


Fig.12. Linearity curve of Alogliptin benzoate

Table.9: Linearity data for alogliptin

S.No	Concentration (ng/spot)	Peak area
1	100	404.85
2	200	600.23
3	300	839.89
4	400	1023.36
5	500	1236.45

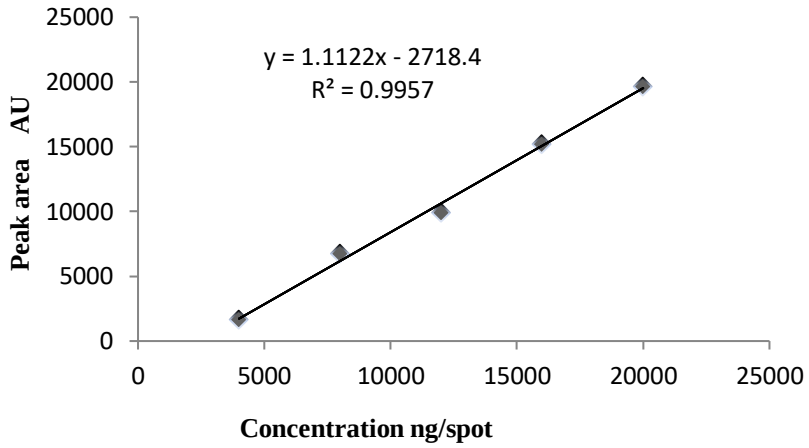


Fig.13. Linearity curve of Metformin hydrochloride

Table.10: Linearity data for Metformin

S.No	Concentration (ng/spot)	Peak area
1	4000	1634.24
2	8000	6742.45
3	12000	9921.56
4	16000	15186.39
5	20000	19656.81

Fig.14. Chromatogram for Alogliptin and Metformin at 254 nm in standard solution

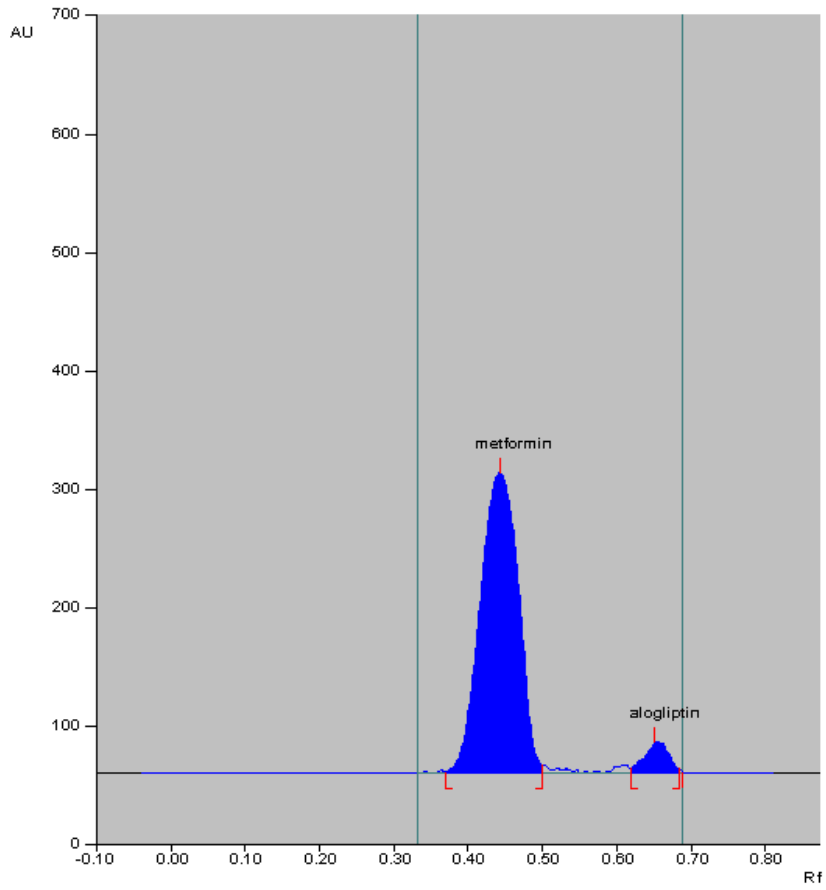
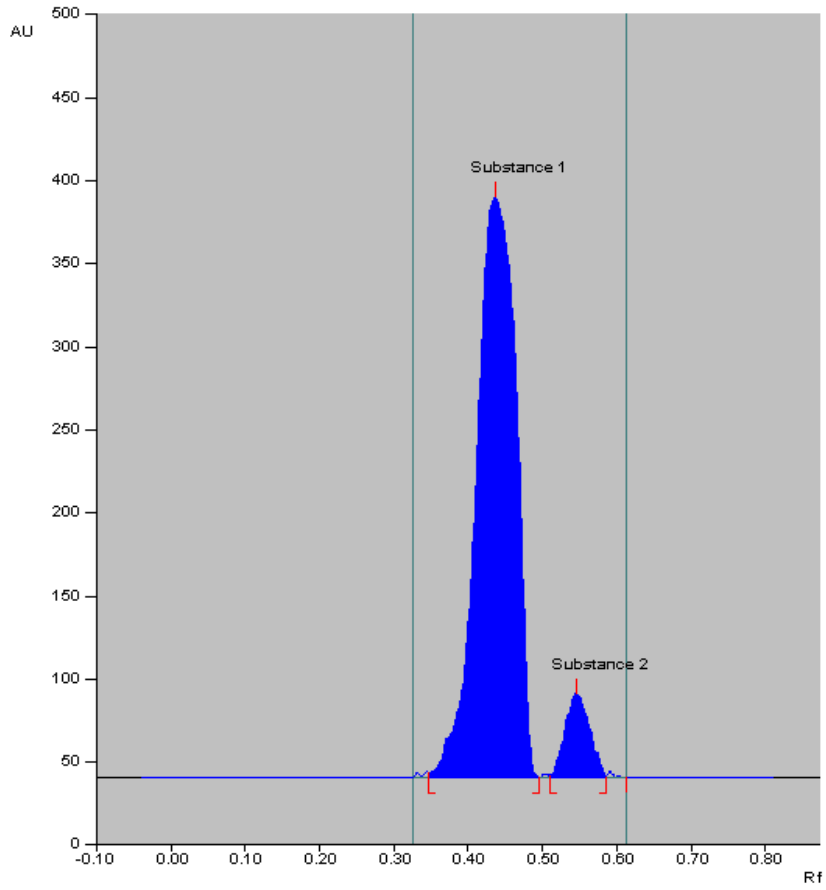


Fig.15. Chromatogram for Alogliptin and metformin at 254 nm in formulation



Chromatogram of all standards

Fig.16.Concentration 1 (metformin: 4000 ng/ml and alogliptin: 100 ng/ml)

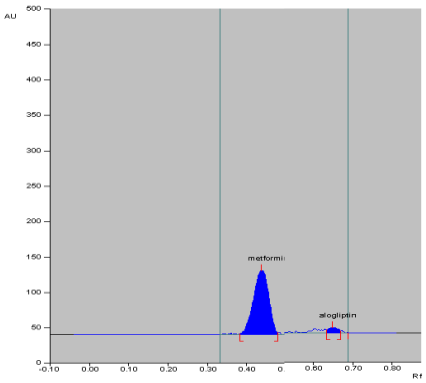


Fig.17. Concentration 2 (metformin: 8000 ng/ml and alogliptin: 200 ng/ml)

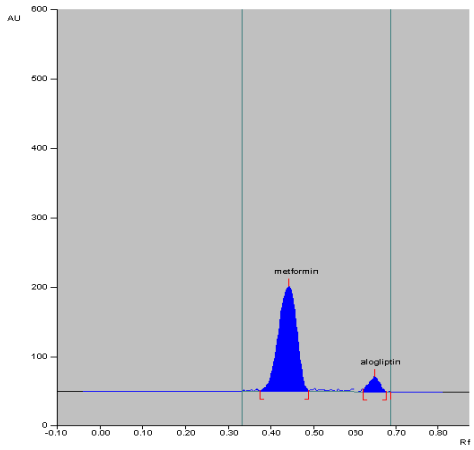


Fig.18. Concentration 3 (metformin: 12000 ng/ml and alogliptin: 300 ng/ml)

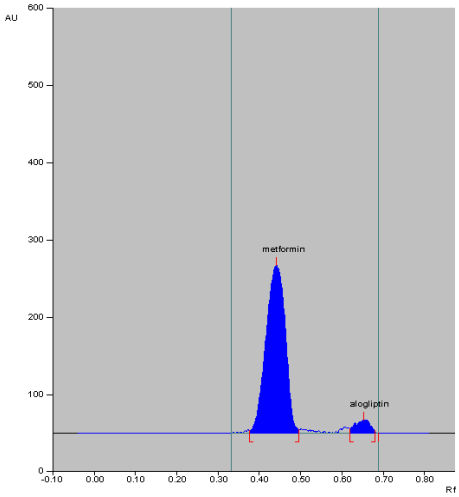


Fig.19. Concentration 4 (metformin: 16000 ng/ml and alogliptin: 400 ng/ml)

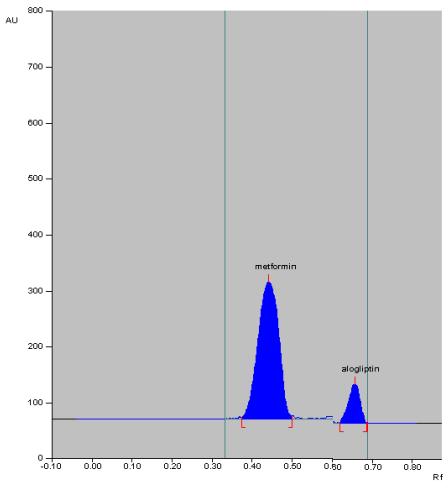


Fig .20.Concentration 5 (metformin: 20000 ng/ml and alogliptin: 500 ng/ml)

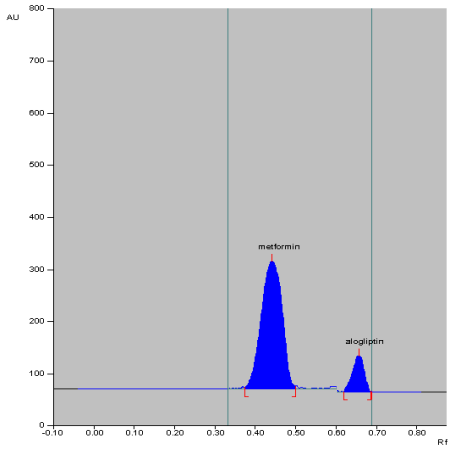


Fig.21. UV Spectrum of Alogliptin

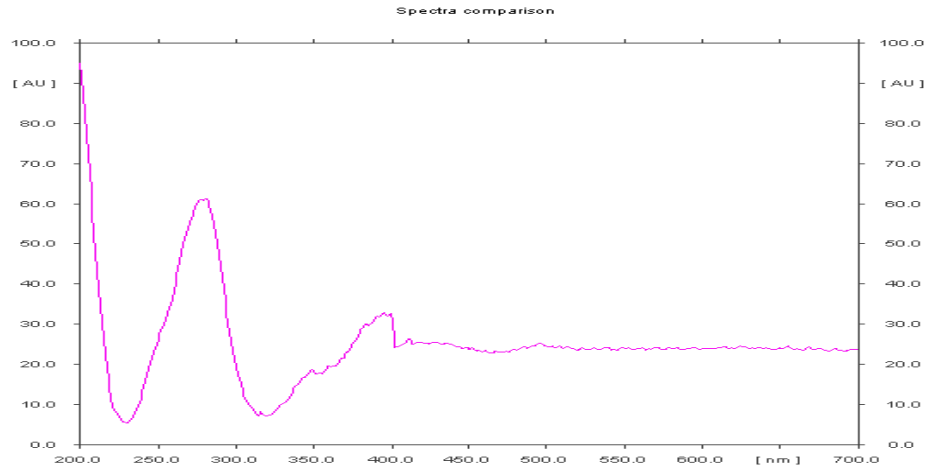


Fig.22. UV Spectrum of Metformin

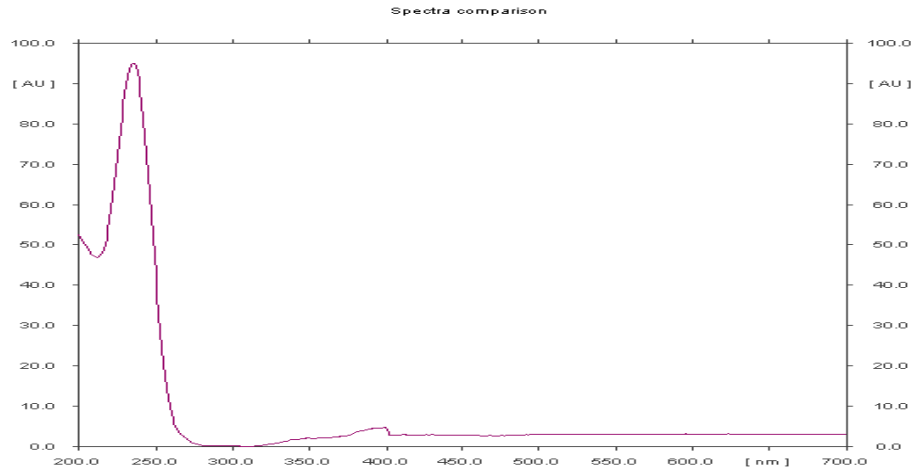


Fig.23. Overlain UV spectrum of metformin and alogliptin

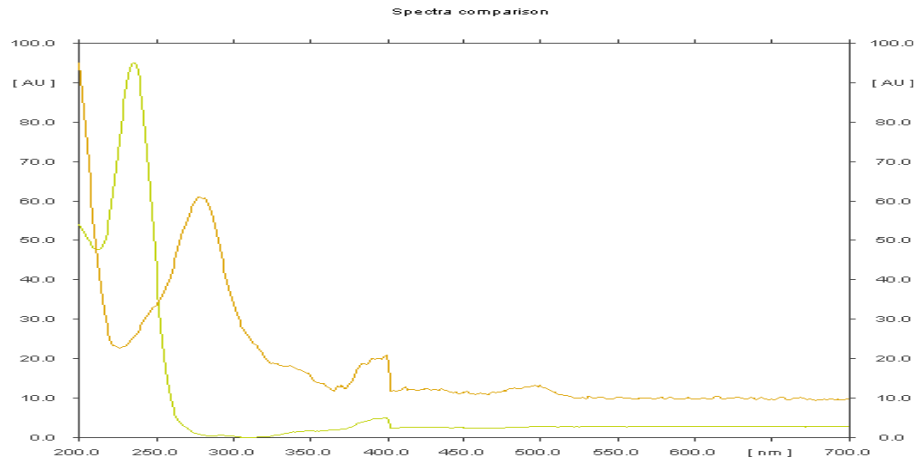
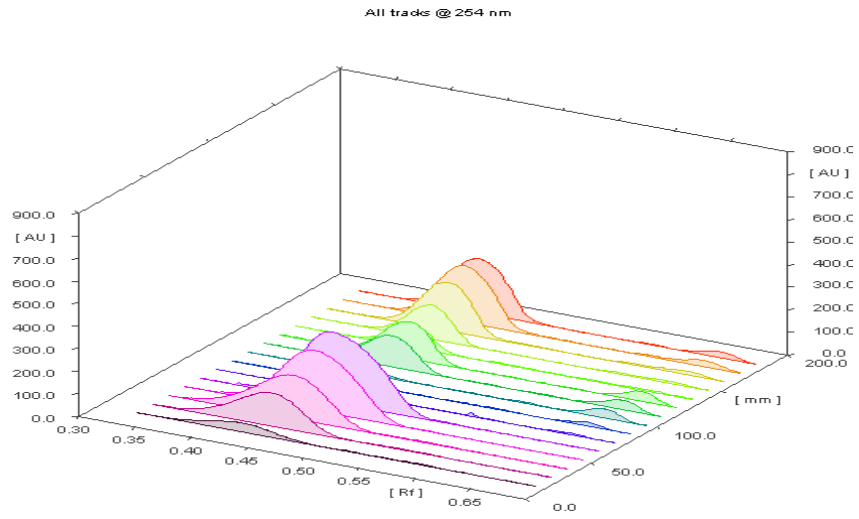


Fig.24. 3D Chromatogram of Alogliptin and Metformin



SIMULTANEOUS QUANTIFICATION OF METFROMIN AND ALOGLIPTIN IN HUMAN PLASMA *(in-vitro*) BY RP-HPLC METHOD

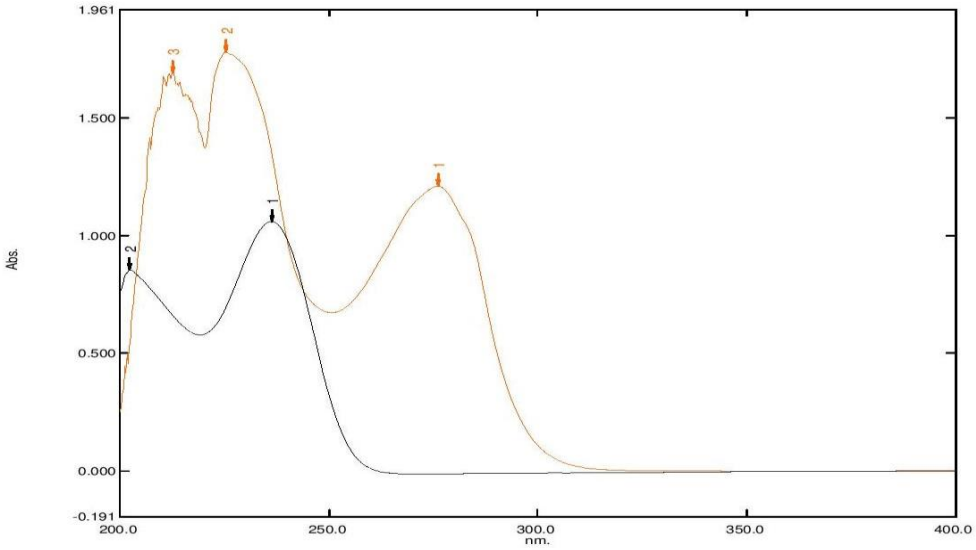
1. **SELECTION OF CHROMATOGRAPHIC METHOD**

   Depending on the nature of the sample (ionic or neutral molecule), its molecular weight and solubility, chromatographic method is to be selected. The drugs are polar in nature so reverse phase chromatographic technique was selected for the present study.

2. **SELECTION OF WAVELENGTH**

   From the UV spectra obtained for both the drugs, 237 nm was selected as the wavelength for the study. **Fig.25.**

   Fig.25: Overlain UV spectrum of Alogliptin and Metformin



3. **OPTIMIZATION OF MOBILE PHASE**

   a) Separation using Methanol (100%)

   Initially 100% methanol was tried as the mobile phase system, only Metformin gave a peak with fronting. But there was no Alogliptin peak (**Fig.26).**

   b) Separation using Methanol: Water

   When tried with slow introduction of aqueous phase with different ratios, Methanol: Water there was no Alogliptin peak **(Fig.27)**.

c) Separation using Acetonitrile: Water

Different ratios of Acetonitrile: Water was tried. In this mobile phase system also there was no Alogliptin peak. Compare to previous mobile phase Metformin gave very broad peak. So Methanol was selected as the organic phase for further studies (**Fig.28).**

d) Separation using Potassium dihydrogen ortho phosphate buffer: Methanol

Potassium dihydrogen ortho phosphate buffer: Methanol (80:20) pH was adjusted to 8 using 30% ammonia solution. In this optimization condition only, Alogliptin gave the peak. But the elution time is more. So different pH was tried. The objective of optimization was to get good symmetrical peaks, well resolved from each other.

In a solvent system consist of Potassium dihydrogen ortho phosphate buffer: Methanol (50:50) pH 8.0 was adjusted using ammonia was fixed as the chromatographic condition for this study **(Fig.29).**

PREPARATION OF MOBILE PHASE

A mixture of methanol: 20 mM potassium dihydrogen ortho phosphate in water (pH-8) (50:50 v/v) was prepared. The pH was adjusted to 8 using 30 % ammonia solution. The resultant mobile phase was degassed in an ultra Sonicator for 15 min.

PREPARATION OF 20 mM POTASSIUM DIHYDROGEN ORTHO PHOSPHATE BUFFER

The phosphate buffer solution was prepared by 2.7218 g of potassium dihydrogen ortho phosphate was dissolved in 1000 ml of purified water in clean and dry flask. The mixture was sonicated for 20 min.

Fig.26. Methanol 100%

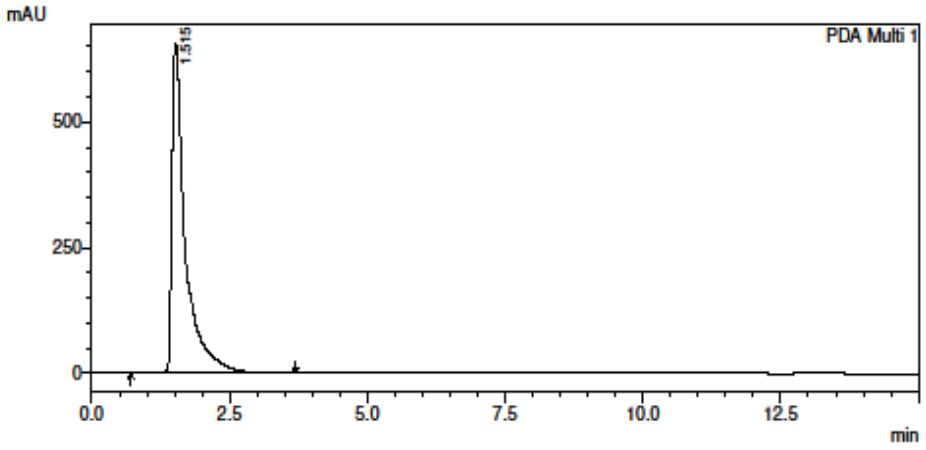


Fig.27. Methanol: Water (20:80)

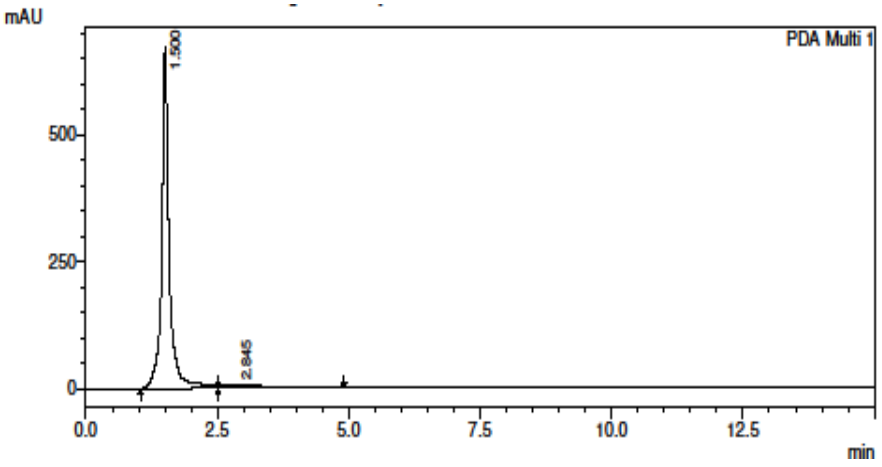


Fig.28 Acetonitrile: Water (20:80)

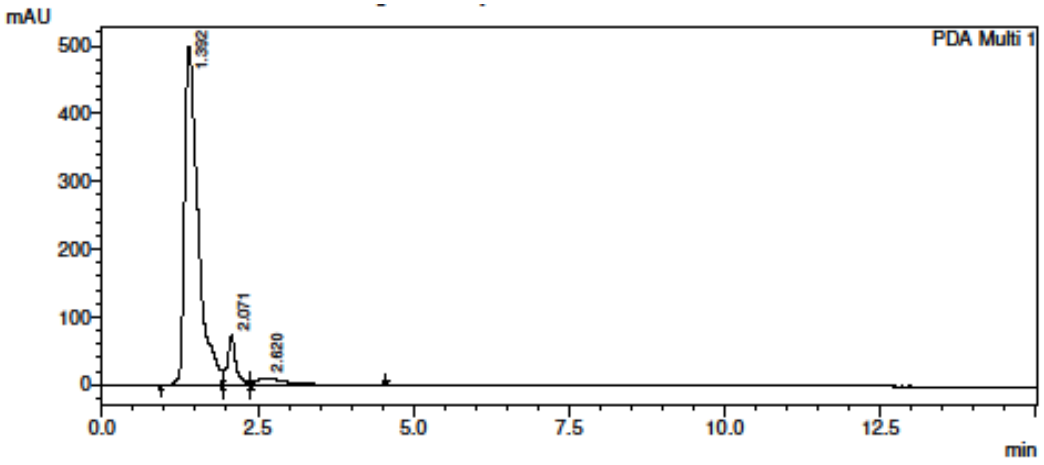
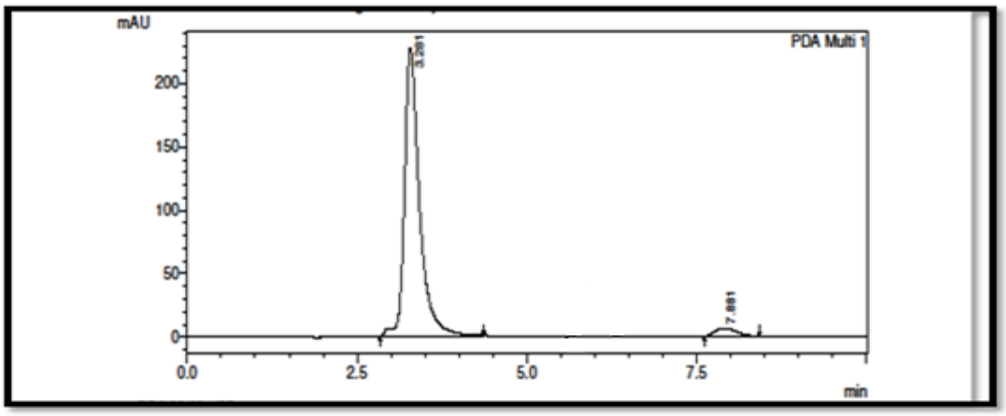


Fig.29 : Potassium dihydrogen ortho phosphate: Methanol (50:50)



OPTIMIZATION OF SEPARATION CONDITIONS

Effect of ionic strength of potassium dihydrogen ortho phosphate buffer

Different ionic strengths like 50 mM, 20mM were tried. The ionic strength of 50 mM peaks are not resolved and showing fronting. Good symmetric peaks were obtained with an ionic strength of 20mM and hence this strength was selected for further studies **(Fig. 30, 31).**

Effect of mobile phase ratio

Mobile phase system consisting of methanol and 20mM potassium dihydrogen ortho phosphate buffer in different ratios like (60:40), (80:20), (50:50) (%v/v) were tried. **(Fig.32-34).** In ratio of (60:40) both the peaks merge into each other. In ratio of (80:20) peak showing more retention time. A ratio of (50:50) gave good symmetrical peak and hence this ratio was fixed.

Effect of pH

The mobile phase methanol and 20mM potassium dihydrogen ortho phosphate buffer in the ratio (50:50) was kept constant and the chromatogram of drug solution were recorded in different buffer pH ranging from 6-8. Good symmetrical peaks were obtained at pH 8.0 which was selected for further studies. (**Fig.35-36)**.

Effect of flow rate

The chromatogram were recorded at different flow rates like 0.3, 0.5, 1.0 mL/min keeping the ratio of mobile phase and buffer pH constant. **(Fig.37-39)**. At a flow rate 0.3 mL/min the peaks are broad and tailing and flow rate 0.5 mL/min peaks resolution is more.

Hence a flow rate 1 mL/min which gave good symmetric peaks with resolution was selected for further studies.

FIXED CHROMATOGRAPHIC CONDITIONS

Stationary phase : Phenomenex Luna C18 (250 x 4.6 mm) 5 µm

Mobile phase : Methanol: 20mM potassium dihydrogen ortho phosphate Buffer pH adjusted to 8 using ammonia

Solvent ratio : 50:50v/v

pH : 8.0

Detection wavelength : 237 nm

Flow Rate : 1.0 mL/min

Retention time

Metformin : 2.9 min

Alogliptin : 7.1 min

EFFECT OF IONIC STRENGTH OF POTASSIUM DIHYDROGEN PHOSPHATE BUFFER ON SEPARATION

Fig. 30: 50mM potassium dihydrogen phosphate buffer

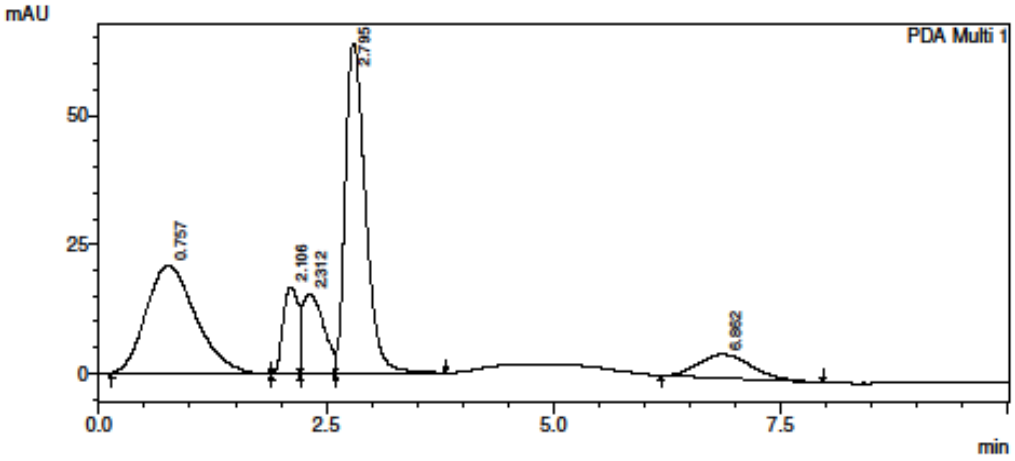
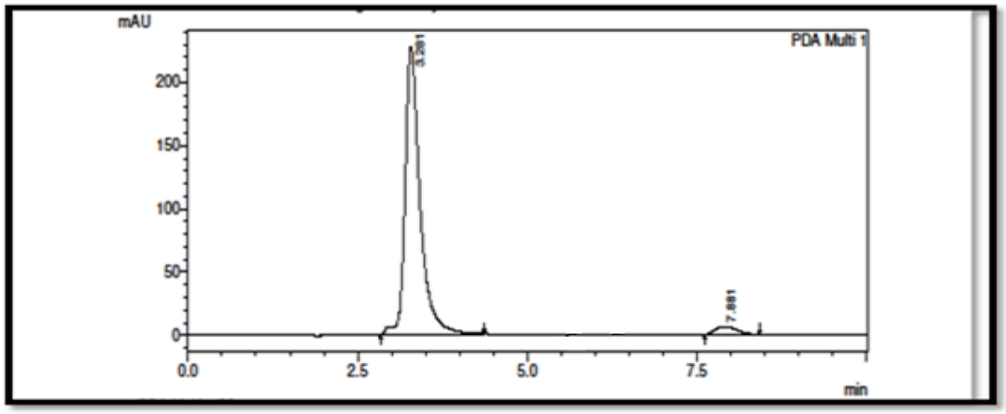


Fig.31: 20mM potassium dihydrogen ortho phosphate buffer



EFFECT OF MOBILE PHASE RATIO

Fig.32: (Methanol: potassium dihydrogen ortho phosphate buffer) 60:40

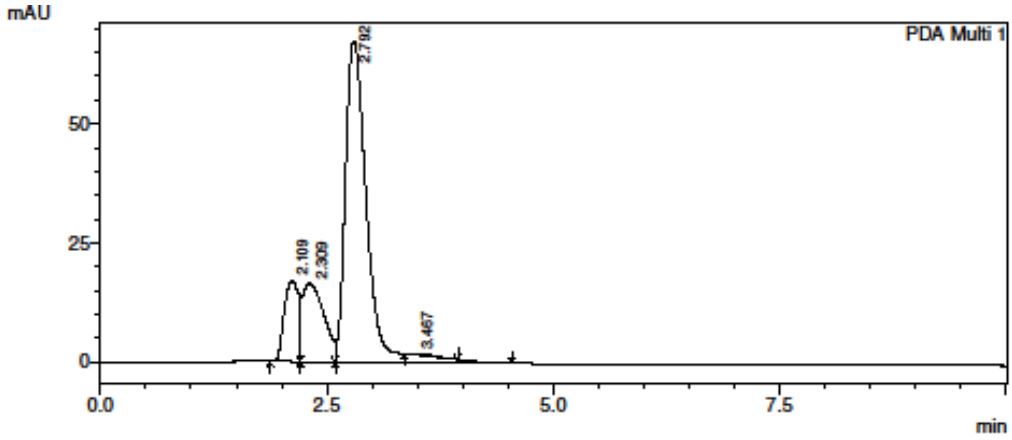


Fig 33: (Methanol: potassium dihydrogen ortho phosphate buffer) (80:20)

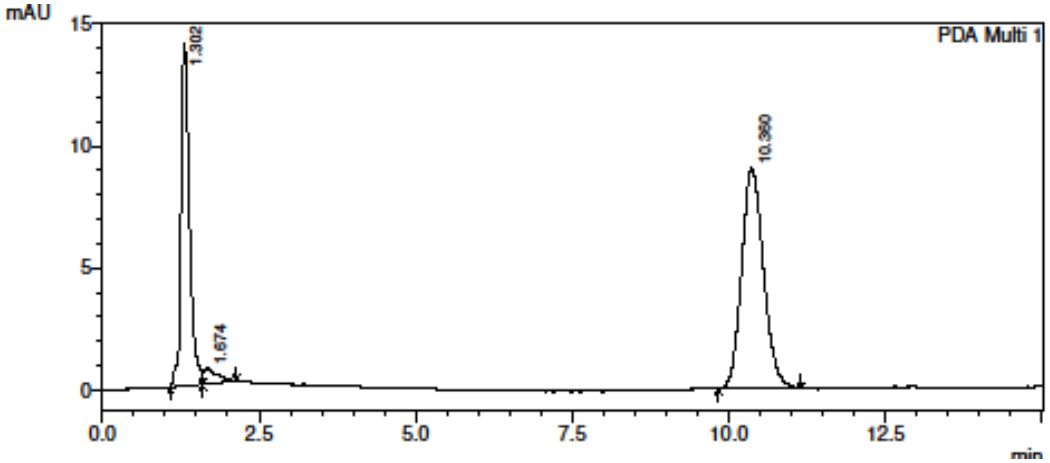
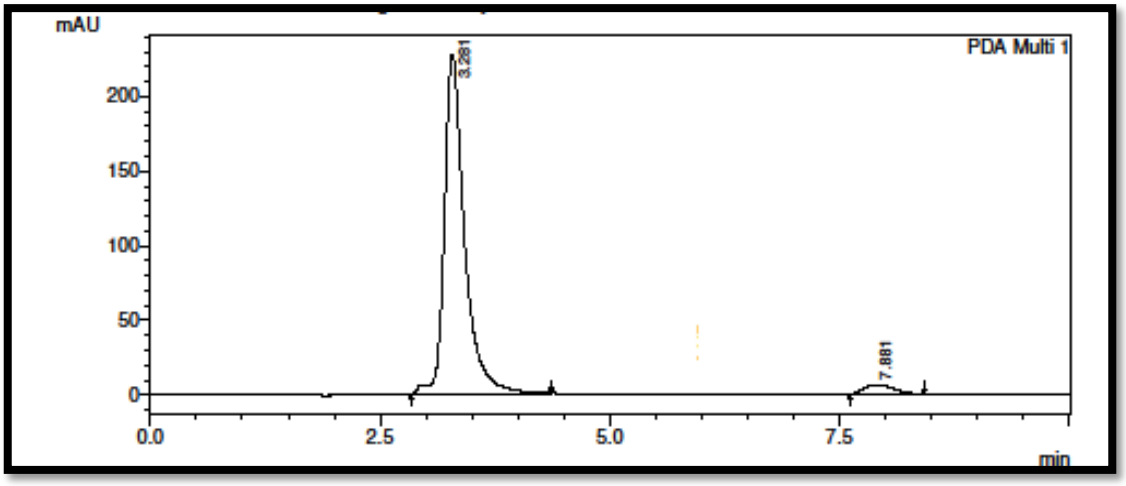


Fig .34.10:50:50 (Methanol: Potassium dihydrogen ortho phosphate buffer) (50:50)



EFFECT OF PH ION SEPARATION

Fig.35: pH 6.0 of potassium dihydrogen ortho phosphate

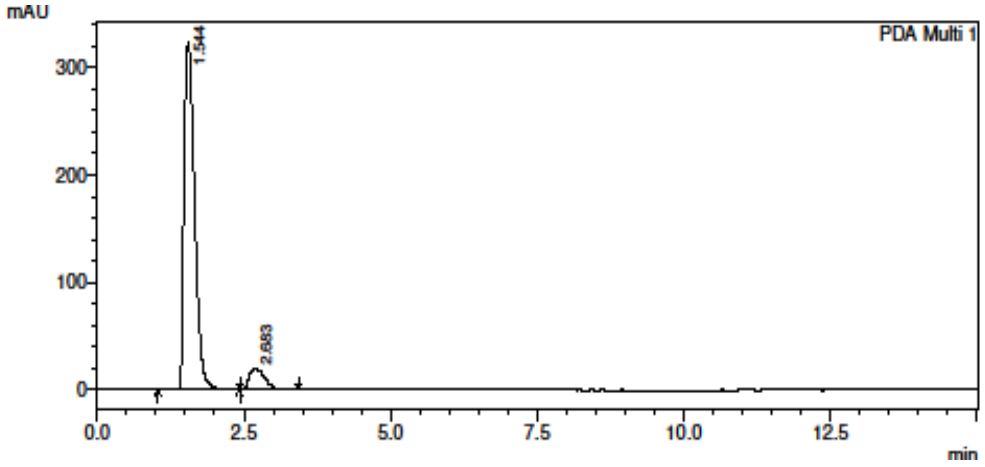
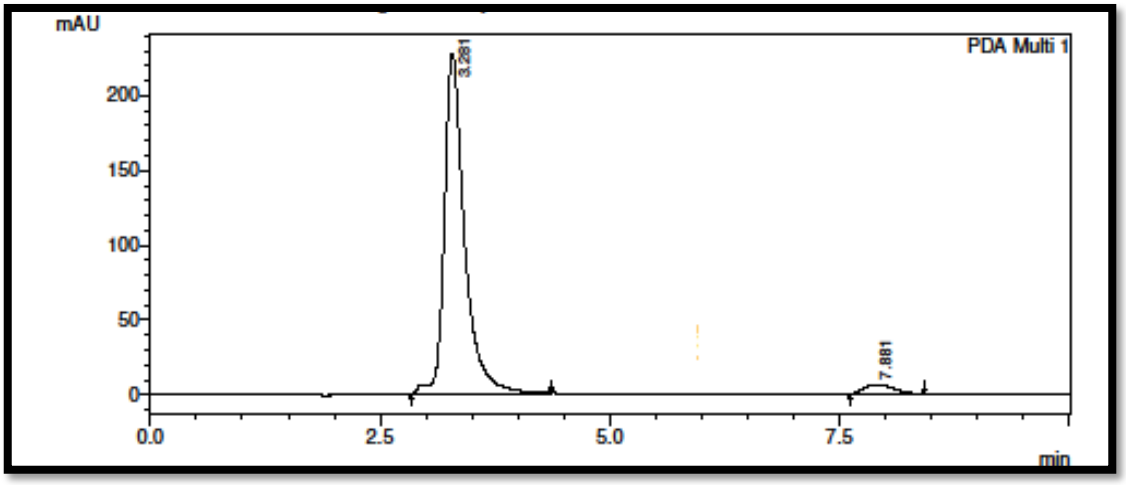


Fig.36. pH 8.0 of potassium dihydrogen ortho phosphate



EFFECT OF FLOW RATE ON SEPARATION

Fig .37: Flow rate 0.3 mL/min

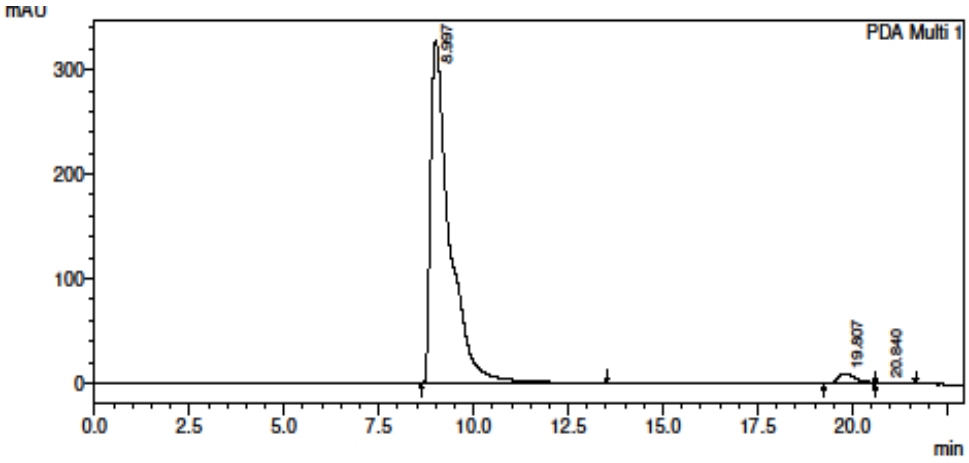


Fig38: Flow rate 0.5 mL/min

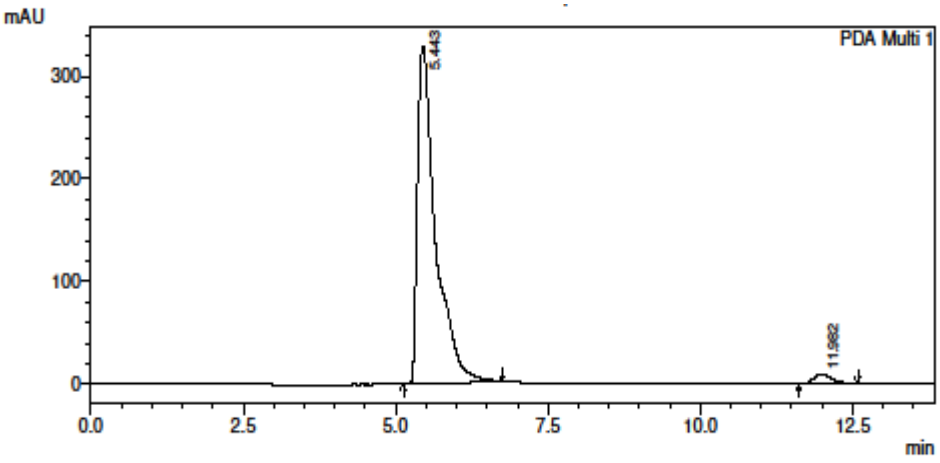
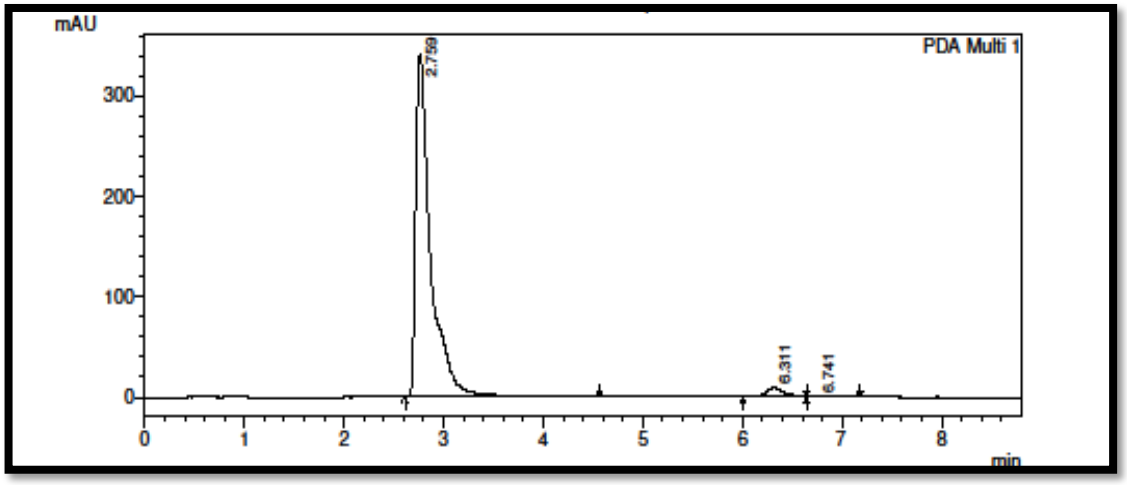


Fig.39: Flow rate 1.0 mL/min



The above developed HPLC method was utilized for *in-vitro* estimation of metformin and alogliptin from plasma.

For extracting the maximum quantity of drug from plasma, the extraction procedure was to be optimized. The main parameters optimized were

1. Extraction method
2. Organic solvent for extraction

SELECTION OF EXTRACTION METHOD

Plasma consists of interferents, which interferes with the quantification of drug. Hence the drug must be separated from interferents. Extraction methods are employed for removal of interfering substances as primary requirement the substances to be extracted must be soluble not only in aqueous biological media but also in a phase which is immiscible with water eg. Dimethyl formamide, Acetonitrile and Methanol.

INITIAL EXTRACTION PROCEDURE

Metformin 200-520 µg/ml and 5-13 µg/ml of Alogliptin, 0.5 ml was pipette out and spiked into 0.5 ml of plasma in polypropylene tube (Torson's). Then the tube was cyclomixed for 5 min. Then the 1 ml of methanol was added to the tube and centrifuged for 20 min at 3000 rpm. Further supernatant liquids were collected in another eppendrof tube and supernatant was injected into the analytical column. The same procedure was followed for the acetonitrile and dimethyl formamide. But in methanol only the drugs gave good separation and symmetric peaks. So methanol used as extracting solvent for further studies.

SELECTION OF ORGANIC SOLVENT FOR EXTRACTION

The extraction of drug into organic solvent depends on its distribution coefficient in aqueous and organic phases. Different solvents like dimethyl formamide, chloroform, acetonitrile, methanol etc. were tried. The drugs were not soluble in chloroform, acetonitrile. Based on the solubility and maximum extraction efficiency methanol was selected as solvent for extraction, **(Fig. 40**).

FIXED EXTRACTION PROCEDURE

Metformin 200-520 µg/ml and 5-13 µg/ml of Alogliptin, 0.5 ml was pipette out and spiked into 0.5 ml of plasma in polypropylene tube (Torson's). Then the tube was cyclomixed for 5 min. Then the 1 ml of methanol was added to the tube and centrifuged for 20 min at 3000 rpm. Further supernatant liquids were collected in another eppendrof tube and supernatant was injected into the analytical column **(Fig 41).**

PREPARATION OF STANDARD SOLUTION

A stock solution was prepared by dissolving accurately weighed 10 mg of metformin and 1 mg of alogliptin in a 10 ml of methanol to yield a concentration of 1000 µg/ml of metformin and 100 µg/ml of alogliptin, respectively. The solution was sonicated for 20 min. Series of aliquots of drug solution containing 200 to 520 µg/ml of metformin and 5 to 11 µg/ml of alogliptin were prepared. To each of five centrifuge tubes 0.5 ml of above prepared solutions was transferred. To this 0.5 ml of plasma was added and with 1 ml of extracting solvent of methanol was added to the tube and centrifuged for 20 min at 3000 rpm. Further supernatant liquids were collected in another eppendrof tube and supernatant was injected into the analytical column.

PREPARATION OF SAMPLE SOLUTION

Fixed dose working sample solutions were prepared according to dosage forms of kazano® tablets. Accurately weighed samples of kazano® contain 500:12.5 mg of metformin and alogliptin were transferred into 10 ml volumetric flask, dissolved in methanol and sonicated for 20 min and filtered the solution and from that aliquots solution are prepared. From the above prepared sample solution (metformin 200-520 µg/ml and 5-13 µg/ml of alogliptin), 0.5 ml was pipette out and spiked into 0.5 ml of plasma in polypropylene tube (Torson's). Then the tube was cyclomixed for 5 min. Then the 1 ml of methanol was added to the tube and centrifuged for 20 min at 3000 rpm. Further supernatant liquids were collected in another eppendrof tube and supernatant was injected into the analytical column. Calibration curve was plotted using peak area of standard drugs Vs concentration of standard solution. The peak area of the sample chromatogram compared and amount of metformin and alogliptin were calculated as shown in **Table.11.** The chromatogram was shown in **Fig. 42.**

CHROMATOGRAM OF BLANK

Blank solution was prepared and chromatographed. It was found that there was no interference from blank plasma **(Fig. 43).**

Table No.11 Analysis of formulation

S.No	Drug	Amount(mg)		%Label claim	%RSD*
		Labelled	Found		
1	MET	500	428.43	85.68	1.2
2	ALO	12.5	10.8	86.4	1.5

*RSD of five observations

VALIDATION OF THE METHOD

LINEARITY

Calibration graphs were plotted following extraction procedure. The spiked samples of plasma were found to be linear in the concentration range from 200-520 µg/ml of metformin and 5-13 µg/ml of alogliptin. The final concentration was found to be in the linearity range of the drugs shown in **(Fig. 44, 45)** and the chromatogram was shown in **(Fig. 46-50).** The calculated statistical data are shown in **Table 12, 13.**

PRECISION

Precision of the method was demonstrated by

1. Intraday precision
2. Interday precision
3. Repeatability

Intraday precision

Intraday precision was found by carrying out the analysis at three different concentrations in the linearity range for three times on the same day. % RSD was calculated and the results are represented in **Table 14.**

Interday precision

Interday precision was found by carrying out the analysis at three different concentrations in the linearity range for three days over a period of one week. % RSD was calculated and the results are represented in **Table 15.**

Repeatability

Standard solution of mixture of drug was injected five times and its % RSD was calculated and the results represented in **Table 16.** The chromatogram was shown in **(Fig. 51).**

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

LOD and LOQ were determined by injecting decreasing amounts of drug. The lowest concentration at which peak is determined is called LOD was found out to be 2 µg/ml for metformin and 0.5 µg/ml for alogliptin.

The lowest concentration at which peak can be quantified is called LOQ was found to be for 5 µg/ml Metformin and 1 µg/ml for Alogliptin.

RECOVERY

The extraction efficiency was explained by recovery studies. Various aliquots of concentration of standard were added to fixed concentration of sample and analysed following the extraction procedure described earlier and amount was calculated. % RSD was calculated and the results are represented in **Table 17.** The chromatogram was shown in **(Fig. 52-53).**

SYSTEM SUITABILITY STUDIES

System suitability parameters like plate number, peak asymmetric factor, capacity factor, selectivity factor, resolution factor are calculated with the help of standard chromatogram **Table 18.**

PEAK PURITY TEST

Peak purity test was performed for the standard drug peaks. Peak purity index values of metformin and alogliptin were found to be 0.9996, 1.0000 respectively. Values close to one indicate the purity of the peaks.

Fig.40 Chromatogram for metformin and alogliptin in acetonitrile

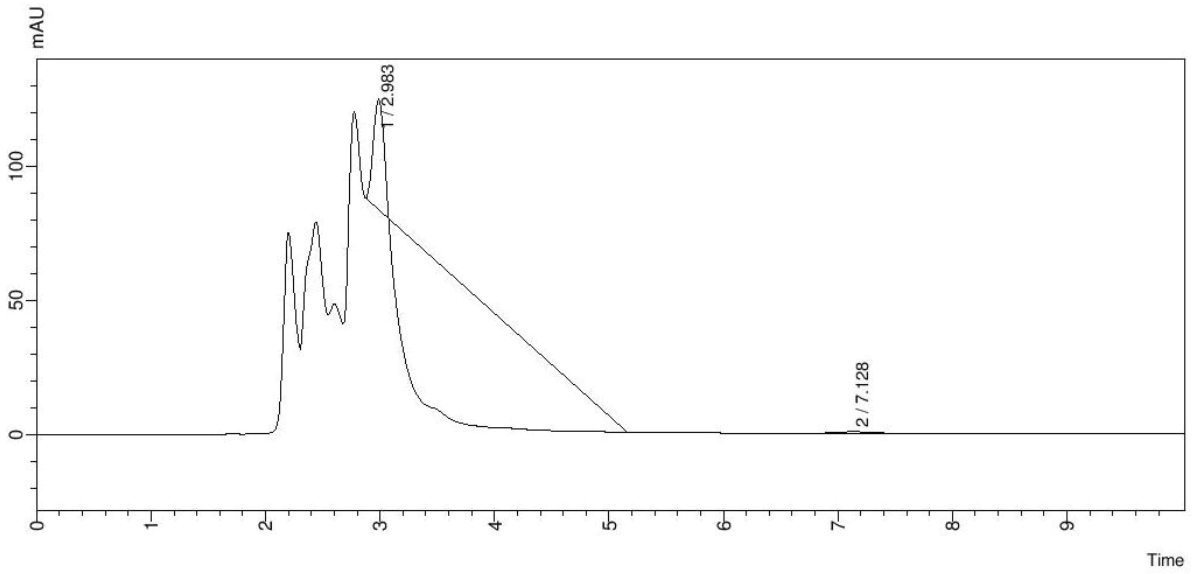


Fig.41 Chromatogram for metformin and alogliptin in methanol

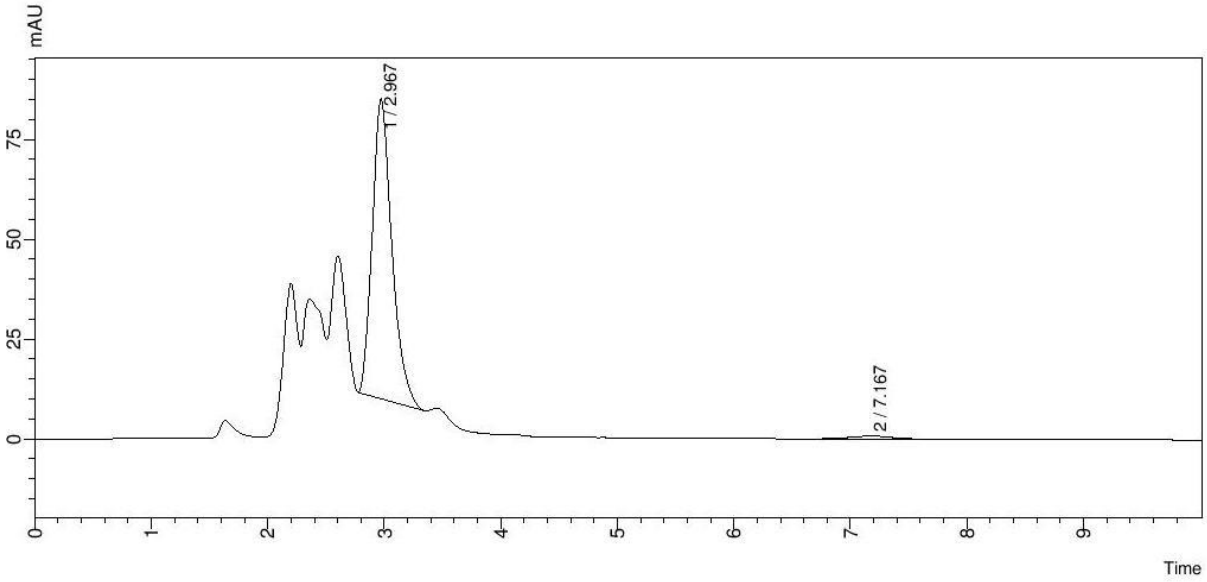


Fig.42 Chromatogram for formulation

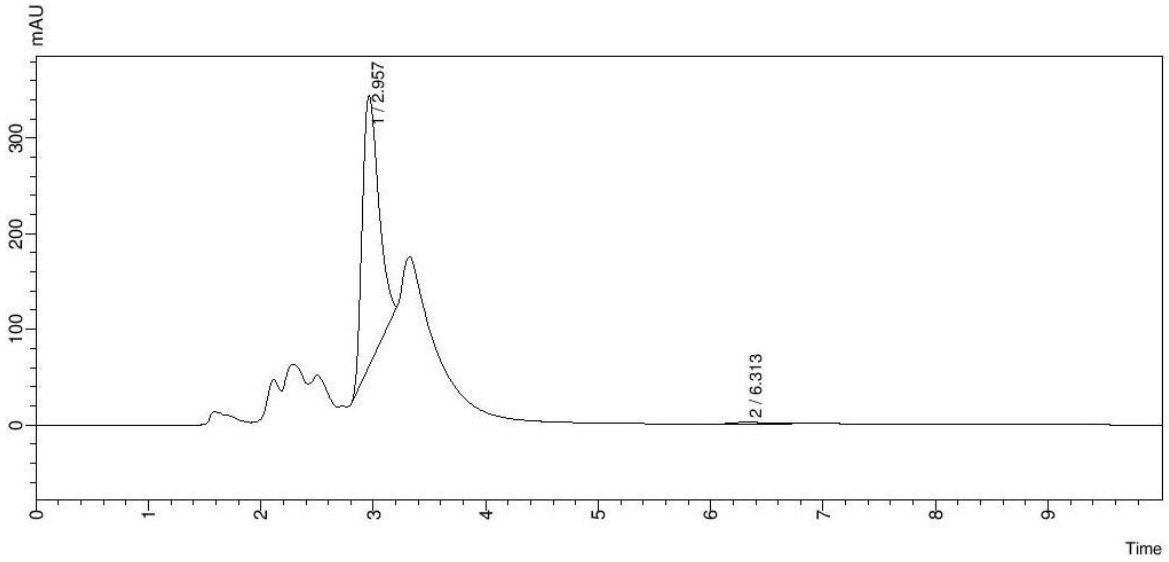


Fig.43 Chromatogram for blank

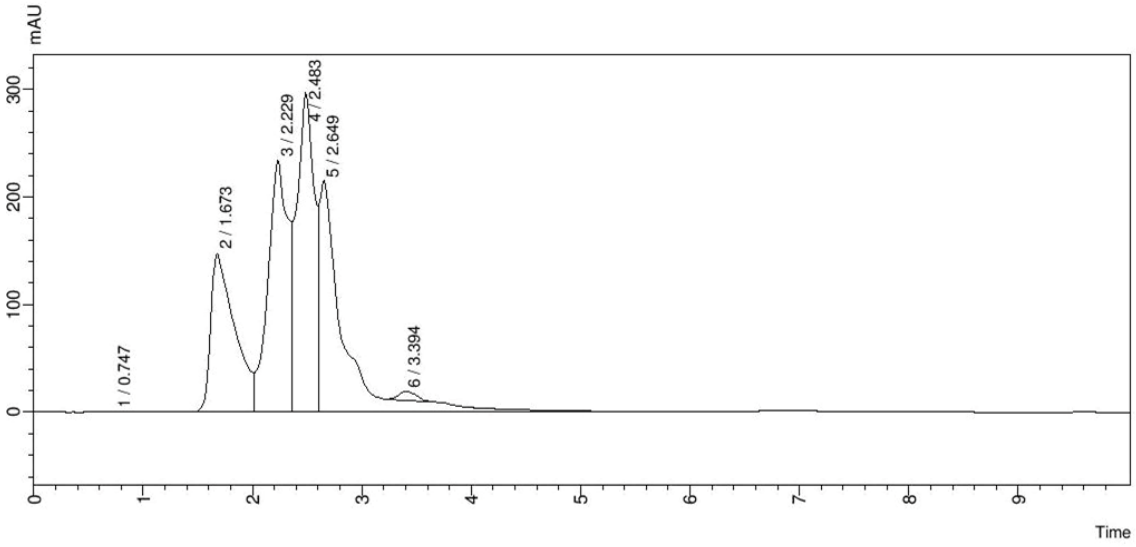


Fig.44 Calibration curve of Metformin

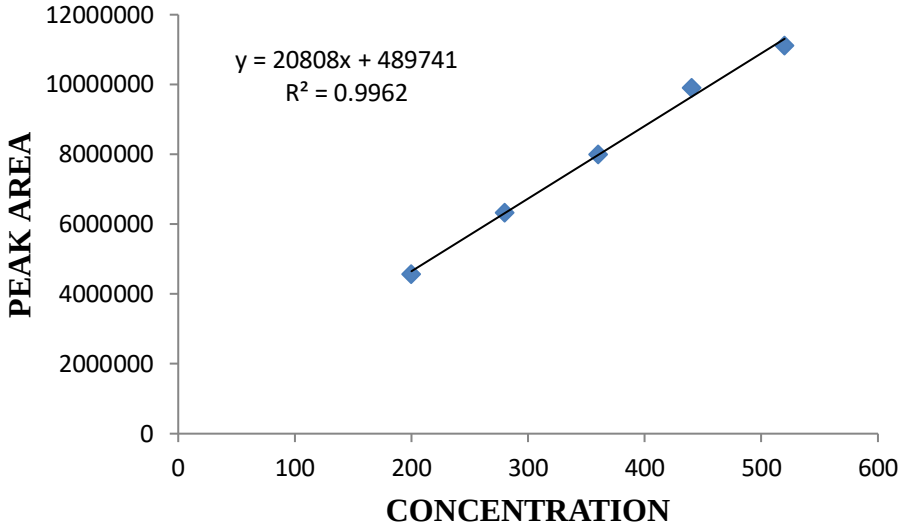


Table 12: Statistical data of the regression equation for Metformin

Parameters	Metformin
Linearity range(µg/mL)	200-520
Coefficient of correlation	0.996
Slope	20808
Y-intercept	48974

Concentration(µg/mL)	Peak area
200	4572766
280	6332815

360	7988601
440	9894137
520	11115454

Fig.44 Calibration curve of Alogliptin

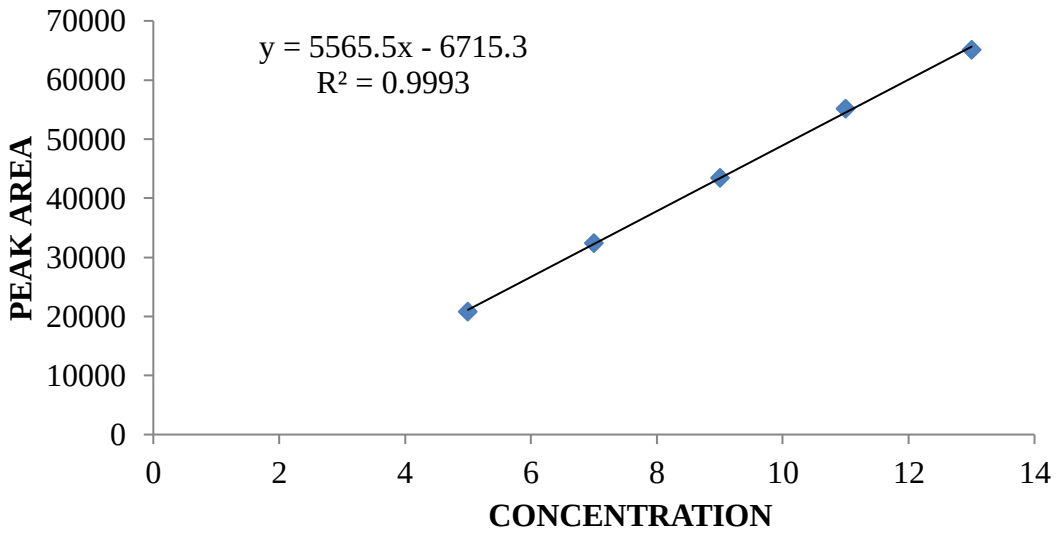


Table 13: Statistical data of the regression equation for Metformin

Parameters	**Alogliptin**
Linearity range(µg/mL)	5-13
Coefficient of correlation	0.987
Slope	5565
Y-intercept	6715

Concentration(µg/mL)	**Peak area**
5	20803
7	32379

9	43479
11	55122
13	65086

Chromatogram of all standards

Fig. 46 Standard 1:

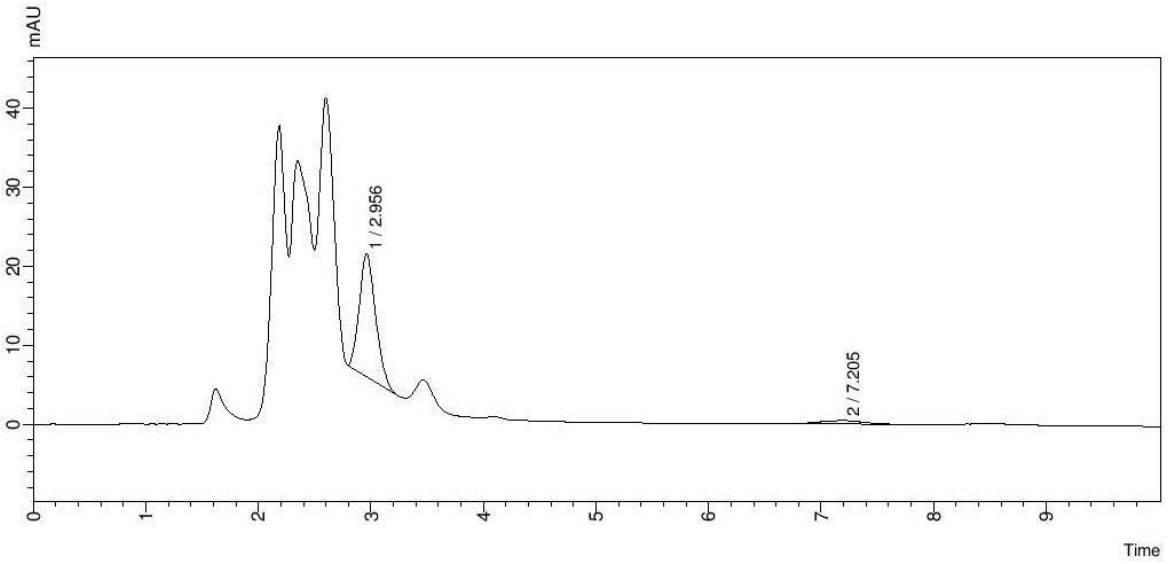


Fig. 47 Standard 2:

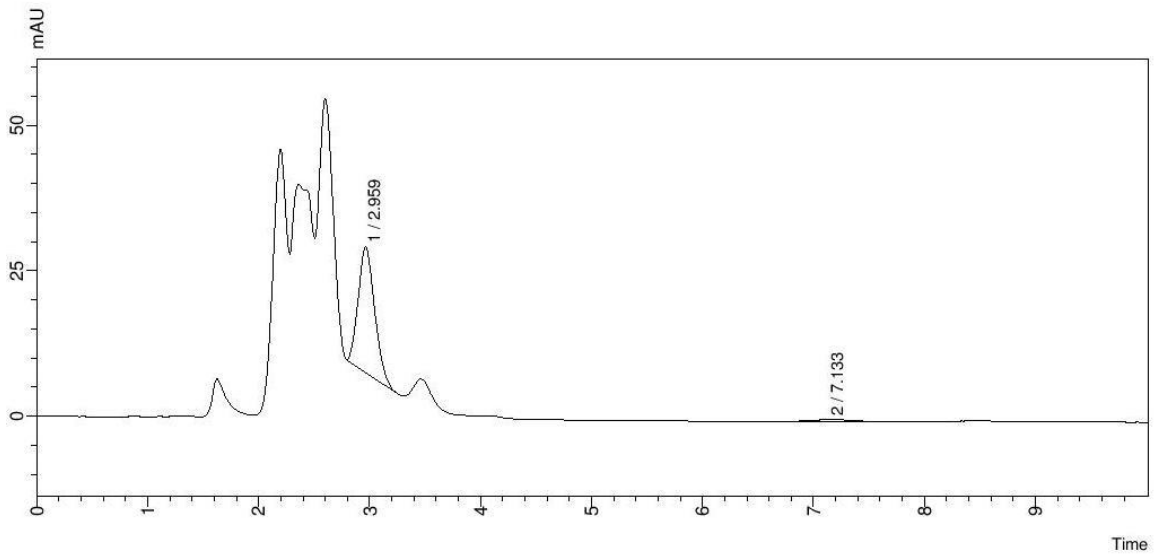


Fig. 48 Standard 3:

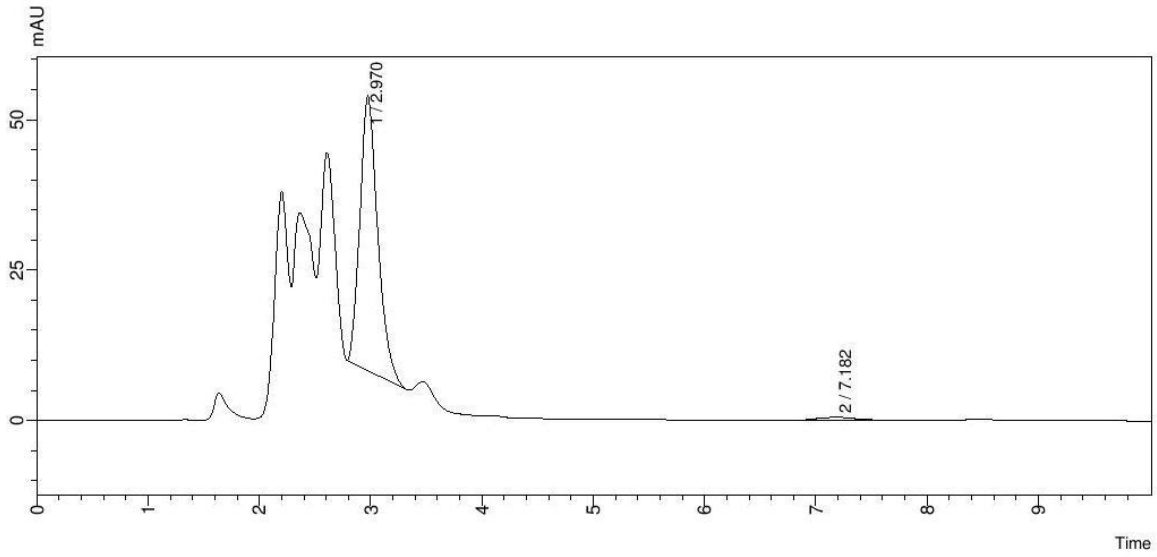


Fig. 49 Standard 4:

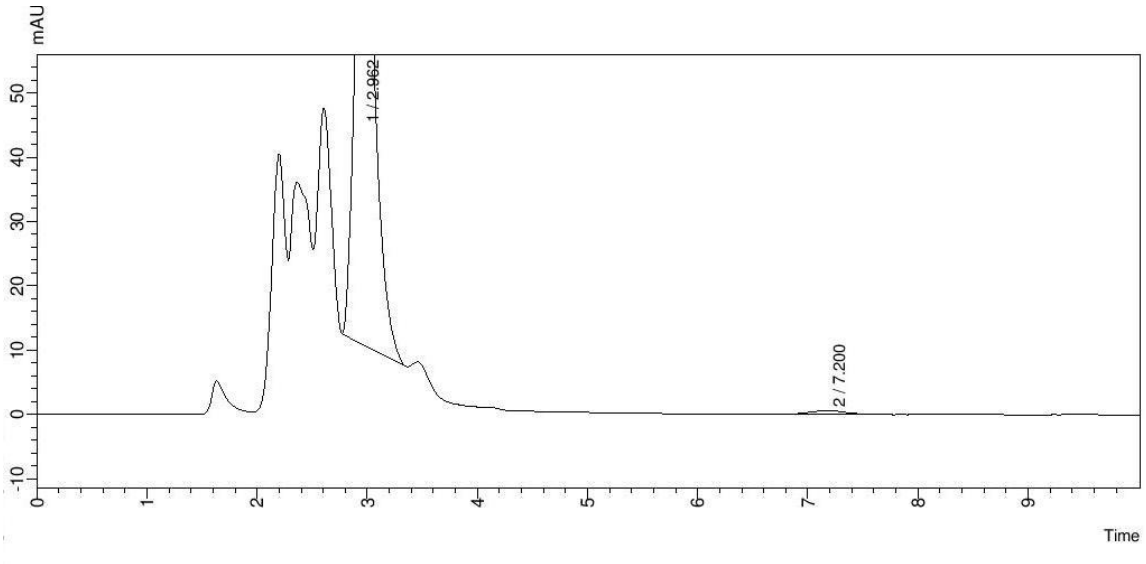


Fig. 50 Standard 5:

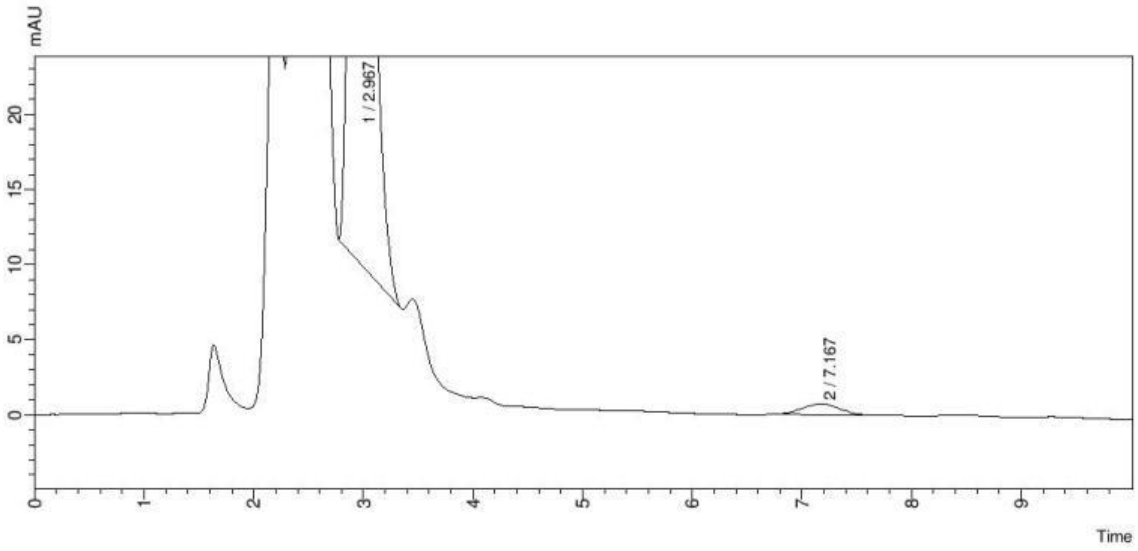


Fig. 51 Repeatability for Metformin

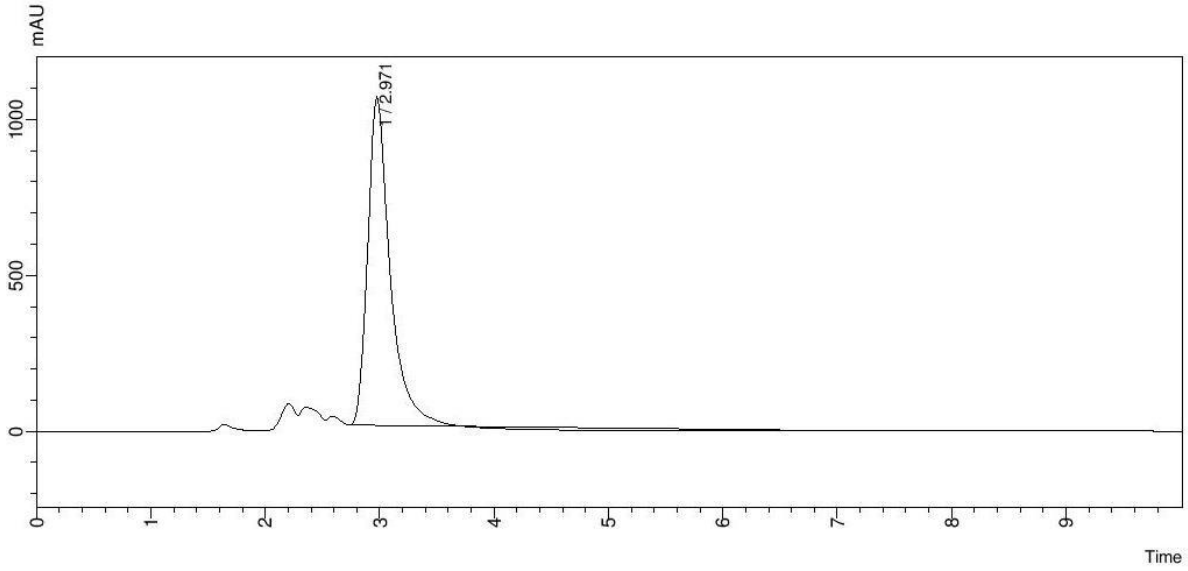


Fig. 52 Repeatability for Alogliptin

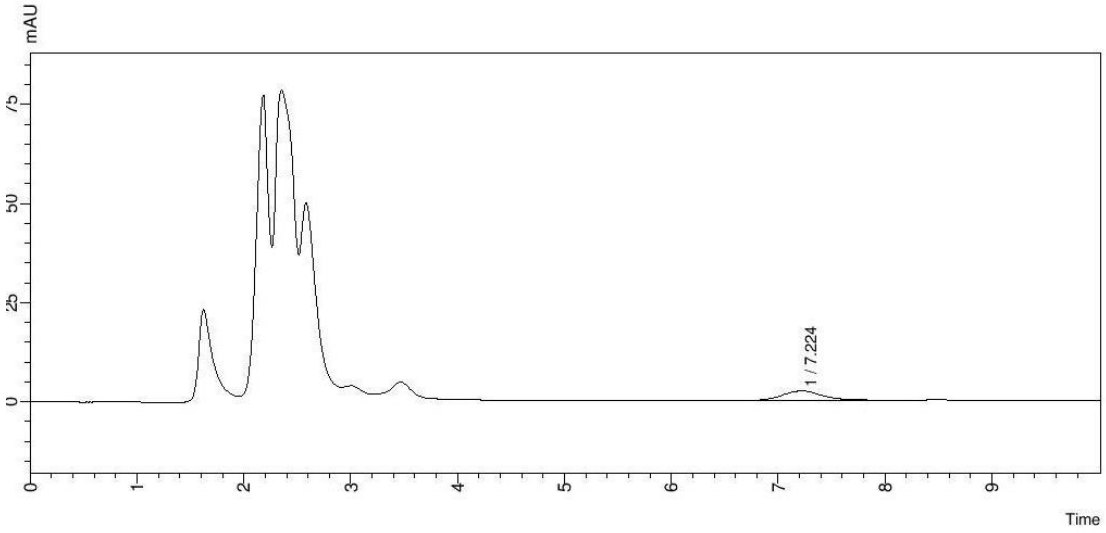


Fig. 53 Recovery for Metformin and Alogliptin

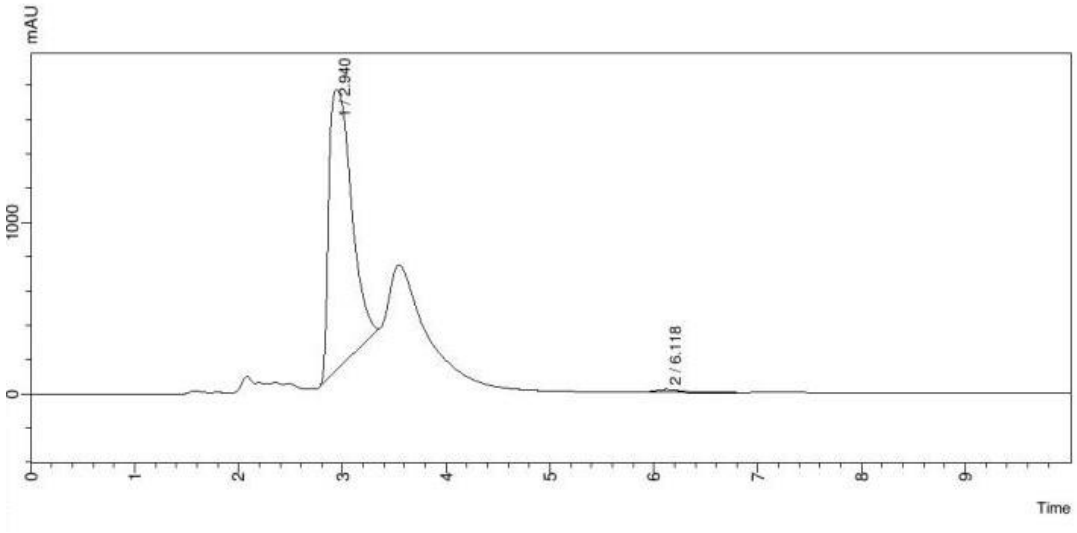


Table 14: Intra-Day precision

Level	Concentration(µg/mL)		Peak area		%RSD	
	MET	ALO	MET	ALO	MET	ALO
I	280	7	657437	35275	0.63	0.72
			650035	34923		
			658090	35421		
II	360	9	726644	41248	0.49	0.32
			720033	41023		
			725832	41009		
III	440	11	943750	53750	0.14	0.79
			945200	53200		
			942520	52920		

Table 15: Inter-Day precision

Day	Concentration(µg/mL)		Peak area		%RSD	
	MET	**ALO**	**MET**	**ALO**	**MET**	**ALO**
1	280	7	675569	25301	0.33	0.71
2			678567	24981		
3			680004	25003		
1	360	9	801534	35701	0.38	0.46
2			805051	35981		
3			798845	35998		
1	440	11	999988	49821	0.68	0.10
2			988985	49881		
3			987643	49781		

Table 16: Repeatability of injection

Concentration (µg/mL)		Injection	Peak area		%RSD	
MET	**ALO**		**MET**	**ALO**	**MET**	**ALO**
200	5	1	3332314	19742	0.78	0.48
		2	3280696	19525		
		3	3332354	19652		
		4	3344789	19555		
		5	3303875	19712		

Table 17. Recovery studies

S.No	Level	% Recovery		% RSD*	
		MET	ALO	MET	ALO
1	80%	98.94	98.9	1.41	0.80
2	100%	101.23	100.6	1.20	0.61
3	120%	99.4	99.78	1.32	0.72

*RSD of three observations

Table 18. System suitability studies

Drug	Rs	N	A
Metformin	1.72	4050.5	1.8
Alogliptin	1.4	3820.7	0.75

RESULTS AND DISCUSSION

The present study was to develop a well validated, sensitive, simple and quick analytical method for the simultaneous determination of metformin and alogliptin in pharmaceutical formulation.

UV spectroscopic method, high performance thin layer chromatographic method was developed for the estimation of metformin and alogliptin in pharmaceutical dosage form. High performance liquid chromatographic method was developed for the estimation of metformin and alogliptin in pharmaceutical dosage form and plasma.

In UV spectroscopic method for the estimation of this combination, methanol was selected as the solvent. The detection wavelength selected was 237 nm for metformin and 225 nm for alogliptin. Linearity showed good linear relationship over a concentration range of 2-10 µg/ml for metformin and the correlation coefficient value was found to be 0.999 for both wavelength and 0.05–0.25 µg/ml for alogliptin and the correlation coefficient value was found to be 0.933 at 237 nm and 0.996 at 225 nm, respectively. It indicates good correlation between the response factor and concentration. The developed method was validated in terms of accuracy, precision and stability. The formulation was extracted with methanol a diluted to get concentration in between the linearity range. Absorbance values were used to determine the amount of drugs in formulation. The drug solution was found to be stable for about 3 hours in room temperature. The results of recovery are represented in **Table 4.**

Estimation of metformin and alogliptin by HPTLC method, methanol was used as the solvent. The wavelength of 254 nm was selected. Mobile phase system consisting of chloroform: methanol: 0.5% ammonium sulphate in the ratio of 4:4:2 % v/v was selected. R_f value of metformin and alogliptin were found to be 0.44 $\pm$0.02, 0.66$\pm$0.02, respectively. The linearity was found to be in the range of 4000- 20000 ng/spot for metformin and 100 – 500 ng/spot for alogliptin, respectively. The correlation coefficient value (0.99) close to 1 proves the linearity. The slope and intercept value were found to be 2.086 and 195 for metformin and 1.112 and 2718 for alogliptin, respectively. The LOD and LOQ was found to be 40 ng/spot and 130 ng/spot for metformin and 2 ng/spot and 6 ng/spot for alogliptin, respectively. Validation parameter like precision, accuracy and stability were carried out and the low % RSD value indicated that the method was good and reproducible. The analyte was

found to be stable for 3 hours. Recovery studies were performed at 80 %, 100 % and 120 % and results are represented in **Table 5.**

Metformin and alogliptin were estimated in plasma by RP-HPLC method. Optimization of chromatographic parameters was carried out by RP-HPLC. Parameters such as selection of chromatographic method for separation, effect of ratio of mobile phase, effect of pH of mobile phase and effect of flow rate were optimised. A mobile phase system consisting of potassium dihydrogen ortho phosphate : methanol was used with the wavelength 237 nm. A mixture of 20 mM potassium dihydrogen ortho phosphate buffer and various ionic strengths of potassium dihydrogen ortho phosphate such as 20 mM and 50 mM were subjected and ionic strength of 20 mM was selected and with 20 mM strength various ratio of mobile phase such as 60:40, 80:20 and 50:50 % v/v was carried out and the ratio of 50:50 was selected. With the above said ratio various buffer pH from 6 and 8 were carried out and pH 8 was selected. Different flow rates were carried out with constant of above mentioned parameters and flow rate of 1 ml/min was selected.

The developed HPLC method was used for the estimation of the combined drugs by in-vitro method. Different extraction solvents were tried like acetonitrile, dimethylformamaide and methanol. Extraction efficient is good in methanol. So methanol is selected as the solvent. Different concentration of drug is spiked with plasma and then the tube was cyclomixed for 5 min. Then the 1 ml of methanol was added to the tube and centrifuged for 20 min at 3000 rpm. The linearity range is 200-520 µg/ml for metformin and 5- 13 µg/ml for alogliptin in plasma, respectively. Extraction method is followed for formulation also. Retention time of metformin and alogliptin were found to be 2.9 and 7.1 min, respectively. Calibration curve was plotted using peak area of standard drugs Vs concentration of standard solution. The peak area of the sample chromatogram compared and amount of metformin and alogliptin were calculated as shown in **Table 11.** Significant percentage of recovery was observed which shows extraction efficiency of the method.

SUMMARY AND CONCLUSION

In this study metformin and alogliptin in combined dosage form was estimated by proposed UV, HPTLC and RP-HPLC method. The main advantages of the proposed methods are its suitability and routine determination of these drugs in combined dosage form.

The fixed dose combination tablet of metformin and alogliptin was subjected to simultaneous estimation by UV spectroscopic method. Compare with the available method, the developed method only analyzed the fixed dose tablet by UV simultaneous equation method. The proposed methods were validated by evaluation of the validation parameters. Assay was performed within a short analysis time.

A new HPTLC method for the simultaneous determination of metformin and alogliptin in pharmaceutical tablet formulation has been developed. The method was found to be sensitive and specific for quantification of metformin and alogliptin in pharmaceutical formulation. The proposed method can be used in detail stability studies of metformin and alogliptin.

The developed HPLC method was used for the estimation of the combination drugs in plasma by *In-vitro* method. Extraction efficiency was determined by the significant recovery studies. The method can be applied for toxicological, bio equivalence, therapeutic drug monitoring studies.

From the forgoing it's concluded that the methods developed are simple, rapid, selective and precise. Hence suitable for application in routine analysis of pharmaceutical preparation and from plasma.

Drug	RP-HPLC		HPTLC		UV	
	% Recovery	Linearity	% Recovery	Linearity	% Recovery	Linearity
Metformin	99.85	200-520 µg/ml	101.01	4000-20000 ng/ml	99.82	2-10 µg/ml
Alogliptin	99.76	5-13 µg/ml	100.20	100-500 ng/ml	98.80	0.05-0.25 µg/ml

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